Developmental regulation of smooth muscle contraction in the urinary bladder

Ekman, Mari

2008

Institutionen för Experimentell Medicinsk Vetenskap
Lunds Universitet

Developmental regulation of smooth muscle contraction in the urinary bladder

Mari Ekman

Akademisk avhandling

som med vederbörligt tillstånd av Medicinska Fakulteten vid Lunds Universitet för avläggande av doktorsexamen i Medicinsk Vetenskap i ämnet Fysiologi kommer att offentligen försvaras i Belfragesalen, Biomedicinskt centrum, Klinikgatan 30, fredagen den 21 november 2008, kl 9.15

Fakultetsopponent
Professor Christian Aalkjaer
Institut for Fysiologi og Biofysik
Aarhus Universitet

LUNDS
UNIVERSITET

DOKUMENTDATABLAD enl SIS 61 41 21

Organization LUND UNIVERSITY Department of Experimental Medical Science	Document name DOCTORAL DISSERTATION Date of issue 2008-11-21 Sponsoring organization FLÄK (Forskarskolan i Läkemedelsvetenskap)
Author(s) Mari Ekman	

Title and subtitle

Developmental regulation of smooth muscle contraction in the urinary bladder

Abstract

Smooth muscle cells in the urinary bladder are involved in the control of urine filling and emptying. We have used mice to study the contractile regulation of urinary bladder smooth muscle during the fetal period. We found that nerve-induced contractions are mainly dependent on muscarinic receptor activation in the newborn whereas adult bladders are equally dependent on muscarinic and purinergic receptor activation. Muscarinic receptor-induced contractions are due to activation of the M3 subtype in both adults and newborns. Activator calcium is mainly recruited from the extracellular space via L-type calcium channels in both adult and newborn tissue. The contractile machinery of the newborn's bladder tissue is more sensitive to calcium which enables contractions at lower intracellular and extracellular calcium concentrations. Non-muscle myosin is contributing to the force development in the newborn but is not the cause for the increased calcium sensitivity. The increased calcium sensitivity is due to a lower phosphatase activity and a lower expression of the myosin light chain phosphatase subunit MYPT1. The contractions induced by muscarinic receptor agonists are characterized by increased phasic activity in the newborn. This activity is not dependent on K+ channels, calcium release from the sarcoplasmic reticulum or gap junctional transmission. Muscarinic receptor stimulation activates T-type calcium channels in the newborn bladder but not in the adult. The main sensitizing pathways, involving protein kinase C (PKC) and Rho-kinase, are established in the newborn tissue. The effects of PKC activation on muscarinic receptor-induced responses differ in newborn and adult tissue. The muscarinic receptor-induced response of the newborn is abolished in the presence of high PKC activation (using PDBu) whereas the response is potentiated in the adult. The phasic component of the response in the newborn bladder is particularly inhibited due to activation of PKC.

Key words: newborn, cellular signaling, muscarinic receptors, calcium sensitivity, myosin light chain phosphatase, protein kinase C, Rho-kinase, non-muscle myosin

Classification system and/or index termes (if any):

Supplementary bibliographical information:		Language English
ISSN and key title: 1652-8220		ISBN 978-91-86059-60-6
Recipient's notes	Number of pages 118	Price
	Security classification	

Distribution by (name and address)

Signature _____ Date 2008-10-13

Don’t fear failure so much that you refuse to try new things.
The saddest summary of a life contains three descriptions:
could have, might have and should have.

Louis Boone

To my family

ISSN 1652-8220
ISBN 978-91-86059-60-6

Lund University, Faculty of Medicine Doctoral Dissertation Series 2008:107

Printed by Media Tryck, Lund, Sweden

CONTENTS

LIST OF PUBLICATIONS

This thesis is based on the following original papers

I Ekman,M., Fagher,K., Wede,M., Stakeberg,K. and Arner,A. Decreased phosphatase activity, increased Ca^{2+} sensitivity, and myosin light chain phosphorylation in urinary bladder smooth muscle of newborn mice. *J. Gen. Physiol.* 125, 187-196 (2005).

II Ekman,M., Andersson,K.E. and Arner,A. Developmental regulation of nerve and receptor mediated contractions of mammalian urinary bladder smooth muscle. *Eur. J. Pharmacol.* 532, 99-106 (2006).

III Ekman,M., Andersson,K.E. and Arner,A. Signal transduction pathways of muscarinic receptor mediated activation in the mouse urinary bladder. *BJU Int*. In press 2008.

IV Ekman,M., Andersson,K.E. and Arner,A. Receptor-induced phasic activity of newborn mouse bladders is inhibited by protein kinase C and involves T-type calcium channels. Manuscript 2008.

ABBREVIATIONS

$[Ca^{2+}]_i$	intracellular calcium concentration
8-Br-cGMP	8-bromo cyclic guanosine monophosphate
ACh	acetylcholine
ATP	adenosine 5'-triphosphate
ATP-γ-S	adenosine 5'-(gamma-thio) triphosphate
BK channel	big/large conductance Ca^{2+} activated K^+ channel
CCh	carbachol
CPI-17	C-kinase dependent phosphatase inhibitor of 17 kDa
DAG	diacylglycerol
IP_3	inositol-1,4,5 trisphosphate
L-type calcium channel	long-lasting calcium channel
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MYPT1	myosin phosphatase targeting subunit (MBS)
NO	nitric oxide
OAB	overactive bladder syndrome
PDBu	phorbol 12,13-dibutyrate
PKC	protein kinase C
PKG	protein kinase G
PLC	phospholipase C
PSS	physiological saline solution
RhoA	Ras homolog gene family, member A
ROCK	Rho-kinase (ROK)
SK channel	small conductance Ca^{2+} activated K^+ channel
SR	sarcoplasmic reticulum
T-type calcium channel	transient type calcium channel

INTRODUCTION

Several vital processes of the body depend on a precise regulation of smooth muscle contraction and relaxation. The various functions of smooth muscles include regulation of blood pressure, digestion of food, control of the airway diameter, labour contractions during childbirth and bladder emptying (i.e. micturition). When the contractile regulation fails or if there are changes in smooth muscle properties, the body homeostasis is altered and pathological processes such as high blood pressure, constipation, asthma, prolonged labour and incontinence can develop. Since the functions and demands in the various tissues are very diverse, the properties of the smooth muscle are specific for each tissue. The variability in smooth muscle properties between tissues is not only present in the adult, but is also seen during development, when the individual smooth muscle organs have to adapt to changes in physiological demands. This thesis is focused on developmental changes of smooth muscle properties of the urinary bladder of mice. Increased knowledge in this field is valuable for understanding basal physiological processes in smooth muscle tissue and might also provide information regarding adaptive changes in bladder disease.

The urinary bladder

The urinary bladder has a spherical shape and is located behind the pubic bone in the lower abdomen. The bladder can be divided into two main components; the bladder base and the bladder dome (Figure 1).

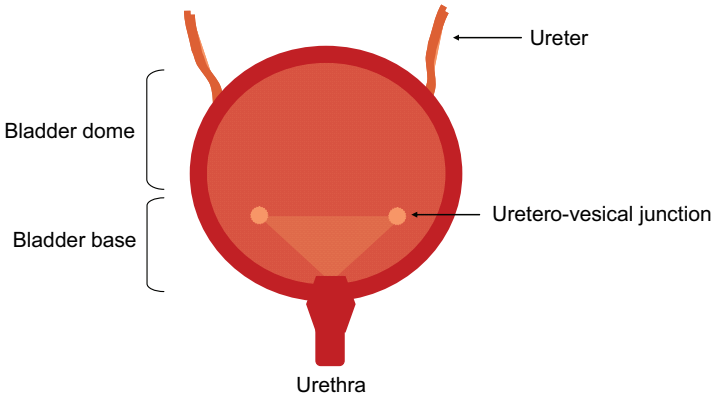

Figure 1. A schematic drawing of the urinary bladder and urethra.

The triangular area between the ureters and the urethral exit is called the trigone and has a supportive role in normal micturition and in preventing the reflux of urine (John et al., 2001). The ureters enter diagonally through the bladder wall and are therefore compressed when the bladder fills. The lower urinary tract contains several interacting smooth muscle tissues; the ureters, the urinary bladder and the urethra. In this complex system the contraction of the smooth muscle is also coordinated with that of the striated muscles. The intraurethral pressure affects micturition and involves both striated and smooth muscles in the wall of the urethra (Andersson, 1993). This thesis focuses on the smooth muscle in the urinary bladder wall.

The wall of the urinary bladder consists of three layers; an inner urothelium with lamina propria, a middle layer of smooth muscles and an outer layer of serosa (Dixon J and Gosling, 1990). The muscular wall of the bladder is composed of the detrusor muscle which consists of bundles of smooth muscles arranged in small functional units (Andersson and Arner, 2004). The ultrastructure of the rat bladder smooth muscle has been characterized in detail in a morphological study (Gabella and Uvelius, 1990). These authors describe that the smooth muscle cells are arranged in bundles, have cellular attachments, and are surrounded by collagen fibrils and have an extensive innervation.

Bladder emptying and filling

Micturition, i.e. bladder emptying and bladder filling are complex physiological events involving neural control, receptor activation and contraction/relaxation of the smooth muscles in the urinary bladder (Andersson and Arner, 2004; Andersson, 1993; de Groat, 2006). One main function of the urinary bladder is storage of urine, which implies that the smooth muscles can relax during the filling phase. The elastic and compliant detrusor muscle allows bladder filling with little or no change in bladder pressure (Abrams, 2003). When the bladder reaches a certain volume, afferent nerve signals are sent to the pontine micturition centre in the brain and initiate a voiding reflex (Chu and Dmochowski, 2006; de Groat, 2006; Morrison et al., 2002). This reflex activates the efferent parasympathetic nervous system and contraction of the detrusor smooth muscles is initiated (Andersson, 1993; de Groat, 2006). The main transmitters in the parasympathetic nerves innervating the bladder are acetylcholine (ACh) and adenosine triphosphate (ATP) (Andersson and Arner, 2004). The active

contraction of the detrusor during micturition is coordinated with a relaxation of the sphincters. Both the internal sphincter, controlled by the autonomous nervous system, and the voluntarily controlled external sphincter, are relaxed and opened for urine to exit the bladder.

Bladder filling and emptying occurs normally throughout life. Physiological conditions, e.g. stress and pregnancy, can be associated with altered micturition patterns. In addition several human pathologies, e.g. the overactive bladder syndrome (OAB), involve significant changes in bladder function, with severe impact on the quality of life for the affected individuals. Without functional urine storage and bladder emptying, newborn animals and children will die if the condition is not treated. The development of the lower urinary tract demands a coordinated growth of the interacting anatomical structures (ureters, bladder, urethra), and of the smooth and striated muscle tissues. Of equal importance is the functional development of the external nervous control systems (sensory and motor nerves), and of the receptors, cellular signaling and contractile machinery in the detrusor. This thesis focuses on these functional aspects in the developing mouse bladder. More information on the developing urinary bladder is given below.

Bladder smooth muscle contraction and relaxation

Extensive information on the regulation of adult bladder smooth muscle contraction is available. Figure 2 illustrates schematically the main activating pathways in the detrusor muscle. These processes are briefly summarized in the sections below.

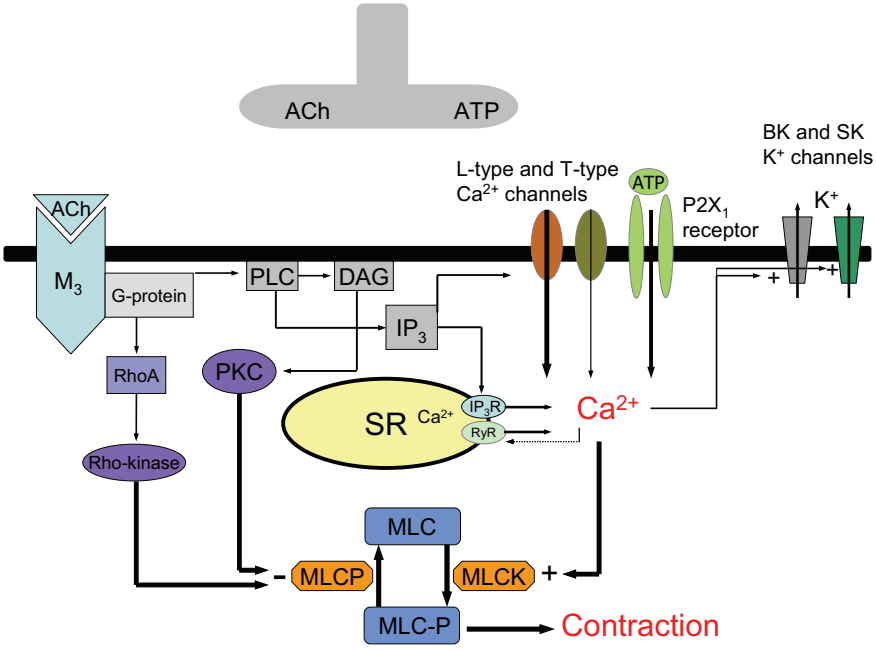

Figure 2. Signaling pathways involved in smooth muscle contraction of the urinary bladder. ACh: acetylcholine, M_3: muscarinic receptor subtype M_3, PKC: protein kinase C, PLC: phospholipase C, DAG: diacylglycerol, IP_3: inositol-1,4,5 trisphosphate, IP_3R: inositol-1,4,5 trisphosphate receptor, RyR: ryanodine receptor, $P2X_1$: purinergic receptor subtype $P2X_1$, MLC: myosin light chain, MLC-P: phosphorylated myosin light chain; MLCK: myosin light chain kinase , MLCP: myosin light chain phosphatase, SR: sarcoplasmic reticulum, BK: large (big) conductance, SK: small conductance, L-type: long-lasting, T-type: transient.

Neural and external control

The urinary bladder is innervated by both sensory and autonomic nerves (Andersson and Hedlund, 2002). The predominant excitatory pathway consists of the parasympathetic nerves with ACh and ATP as the main transmitters (Morrison et al., 2002). Several other excitatory and relaxant substances (e.g. tachykinins, nitric oxide, vasoactive intestinal polypeptive) can be released locally from cells or sensory nerves in the bladder wall (Andersson, 1993; Smet et al., 1996). In addition to the neural control, detrusor smooth muscle contraction is modulated by several hormones and physical/chemical factors e.g. estrogen, stretch, $[H^+]$, temperature (Kanai et al., 2007; Mustafa and Thulesius, 1999; Schultens et al., 2004; Uvelius et al., 1990).

Membrane receptors

The muscarinic receptors (subtypes M_1-M_5) belong to the family of heterotrimeric G-protein coupled receptors (Caulfield and Birdsall, 1998). Initiation of contraction of the detrusor muscle is mainly mediated by activation of muscarinic receptors, predominantly subtype M_3 in adult bladders (Andersson and Arner, 2004; Schneider et al., 2004b; Schneider et al., 2004a; Eglen et al., 1994). Muscarinic M_2 receptors are also present, but the functional role of these receptors is unclear (Abrams et al., 2006; Eglen et al., 1994; Eglen et al., 1996). It has been suggested that M_2 receptors enhance M_3 receptor-mediated contractions (Ehlert et al., 2005) and that M_2-induced contractility is increased in pathologies such as bladder hypertrophy (Ruggieri, Sr. and Braverman, 2006). The M_3 receptor is coupled to the G-protein $G_{q/11}$, which activates phospholipase C (PLC) and induces inositol trisphosphate (IP_3) production and mobilization of intracellular Ca^{2+} (An et al., 2002; Caulfield and Birdsall, 1998). In addition, diacylglycerol (DAG) is produced via PLC activation. The $G_{q/11}$ protein is also coupled to activation of small G-proteins, including RhoA (Ras homologue G-protein A), and activation of protein kinase C (PKC) via DAG. The effects of the PKC and the Rho-kinase (ROCK) pathways on the Ca^{2+} sensitivity of the contractile filaments are discussed below. The M_2 receptor is coupled to the G-proteins G_i or G_0 and is thought to inhibit adenylyl cyclase production (Caulfield and Birdsall, 1998).

Part of the nerve-mediated contractile response in the urinary bladder is resistant to atropine and studies on this component of contraction have shown that ATP is an important transmitter in these responses (Andersson, 1993). The relative contribution of the ATP pathway appears to vary between species. In man ATP-effects appear to be less prominent than in rodents (Sjogren et al., 1982; Kennedy et al., 2007). Interestingly, the purinergic transmission is thought to be affected by diseases such as bladder urinary outlet obstruction, bladder overactivity and inflammation (Andersson and Arner, 2004). The $P2X_1$ receptors bind ATP and belong to a distinct family of ligand-gated cation channels (Roberts et al., 2006). Activation of this receptor in the detrusor muscle induces a rapid phasic contraction due to influx of Ca^{2+} (Sjuve et al., 1995). Opening of the $P2X_1$ receptor-linked channel thus provides Ca^{2+} both for initiation of contraction and for depolarization of the cell (Vial and Evans, 2000).

In addition to the main transmitters (ACh and ATP), the bladder smooth muscle reacts to a large number of substances affecting contraction and relaxation via activation of both sensory and motor neurons (Andersson and Hedlund, 2002; Michel and Barendrecht, 2008). These substances include bradykinin, tachykinins and prostanoids.

Calcium influx and release

In the urinary bladder, activator calcium is recruited via influx through L-type (long-lasting) channels and by release from sarcoplasmic reticulum (SR) (Andersson and Arner, 2004). Ca^{2+} release from the SR in smooth muscle is triggered by IP_3 and by Ca^{2+} (Morrison et al., 2002). However, the role of Ca^{2+}-release from the SR following muscarinic receptor activation appears to be minimal in the bladders of most species. In the mouse, rat, rabbit and human, calcium influx via membrane calcium channels is found to be essential for bladder contraction in responses following muscarinic receptor stimulation (Schneider et al., 2004b; Wegener et al., 2004). Mice deficient in an L-type Ca^{2+} channel subunit (α_{1c} subunit, (Wegener et al., 2004)) have dramatically impaired bladder contractility, suggesting a key role of this channel in detrusor activation. Although Ca^{2+} release from the SR does not contribute to the contractile activation, it might have other functions, e.g. feedback regulation of membrane K^+ channels (Herrera and Nelson, 2002). Another pathway for calcium entry is via T-type (transient) calcium channels which are present in the detrusor layer. They coexist with both L-type calcium channels and calcium activated K^+ channels (Fry et al., 2006). Hyperpolarization increases T-type calcium channel availability. Influx of calcium through these channels may locally activate L-type calcium channels and increase intracellular calcium concentration (Fry et al., 2006).

K^+ channels

Urinary bladder contractility in mice is influenced by small (SK) and large (BK) conductance K^+ channels (Herrera et al., 2005; Meredith et al., 2004; Thorneloe et al., 2008; Werner et al., 2006). These channels are activated by an increase in intracellular [Ca^{2+}] (BK and SK channels) or by membrane depolarization (BK channels) and function as negative feedback regulators of urinary bladder contractility (Herrera et al., 2005; Thorneloe et al., 2008). The BK channels have been suggested to be a part of a negative feedback mechanism, where Ca^{2+}-release

from the SR opens the Ca^{2+} activated BK channels, hyperpolarizes the membrane, and promotes relaxation (Herrera and Nelson, 2002). Both SR and BK channels appear to be essential for this mechanism. Loss of ryanodine receptor function in the SR of rats is correlated with overactive bladder contractions (Jiang et al., 2005). Ablation of the BK membrane channels in mice leads to overactive bladder and incontinence (Meredith et al., 2004).

Cellular regulation of contractile filaments

Intracellular calcium ($[Ca^{2+}]_i$) is the main regulator for the initiation of smooth muscle force. Ca^{2+} binds to calmodulin and this complex activates the myosin light chain kinase (MLCK) (Horowitz et al., 1996). MLCK phosphorylates the regulatory light chains of the myosin molecule. The activated myosin interacts with the actin filaments and generates force and shortening. Relaxation is induced when the myosin light chain phosphatase (MLCP) dephosphorylates the regulatory light chains. Thus the extent of regulatory light chain phosphorylation and force results from a balance between MLCK and MLCP activities (Somlyo and Somlyo, 2003).

Calcium sensitivity of contraction

Although variation in free $[Ca^{2+}]_i$ is the main regulator of smooth muscle force via activation/deactivation of the calcium-calmodulin-MLCK pathway, many agonists can increase force without changes in $[Ca^{2+}]_i$. This phenomenon is called calcium sensitization (Figure 3) (Somlyo and Somlyo, 2003). In experimental situations, it is also possible to examine the relation between the extracellular $[Ca^{2+}]$ and force. The Ca^{2+} sensitivity under these conditions is determined by the properties of the Ca^{2+} influx pathways (L-type Ca^{2+} channels) and the intracellular Ca^{2+} sensitivity. An important factor determining the intracellular Ca^{2+} sensitivity is the MLCK/MLCP activity ratio. The MLCK activity is dependent on the free $[Ca^{2+}]_i$, but changes in its activity independent of $[Ca^{2+}]$, will change the Ca^{2+} sensitivity of the activation and shift the relation between $[Ca^{2+}]$ and myosin phosphorylation. Several kinases phosphorylate MLCK (Somlyo and Somlyo, 2003). One example is the CaM-kinase II (Ca^{2+}/calmodulin dependent kinase II) which has been shown to phosphorylate MLCK, resulting in calcium desensitization during sustained contractions in tracheal smooth muscle (Tansey et al., 1994).

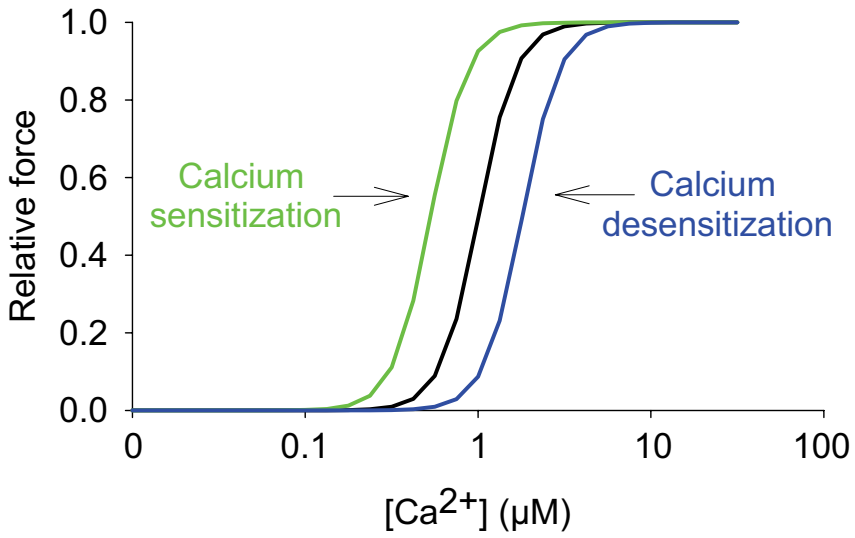

Figure 3. ***Calcium sensitization****: an increased force due to a decrease in MLCP activity or increased MLCK activity at each* $[Ca^{2+}]_i$ *.* ***Calcium desensitization****: a decreased force due to an increase in MLCP activity or decreased MLCK activity.*

The MLCP is an important target for modulation of the Ca^{2+} sensitivity after agonist activation in smooth muscle (Somlyo and Somlyo, 2003). The MLCP consists of three subunits; the catalytic subunit PP1c (37 kDa), the MYPT1 (myosin phosphatase targeting subunit, 110-130 kDa) and the M20 (20 kDa) subunit with unknown function (Ito et al., 2004). The activity of the MLCP can be regulated by phosphorylation of the subunit MYPT1. Two main phosphorylation sites (Thr696 and Thr853 in humans) in MYPT1 are identified (Ito et al., 2004). The Thr853 site appears to be ROCK specific and is phosphorylated in smooth muscle in response to agonist-induced activation (Kitazawa et al., 2003), suggesting that this target is involved in the small G-protein-dependent inhibition of MLCP activity and in Ca^{2+} sensitization. A parallel or alternative pathway, for MLCP inhibition is via CPI-17 (C-kinase dependent phosphatase inhibitor of 17 kDa) (Mizuno et al., 2008; Somlyo and Somlyo, 2003). An important kinase for CPI-17 phosphorylation is PKC (Kitazawa et al., 1999). Several types of cross-talk between CPI-17/MYPT1 and PKC/ROCK seem to exist. ROCK has been shown to phosphorylate CPI-17 (Pang et al., 2005) and PKC has been suggested to directly affect phosphorylation of MYPT1 in the mouse urinary bladder

(Mizuno et al., 2008). It should also be noted that PKC has several other targets in the smooth muscle cell, apart from the proteins involved in the calcium sensitization, e.g. L-type calcium channels (Lin et al., 1998). Most of calcium sensitization mechanisms described above have been identified in the detrusor smooth muscle. Studies on urinary bladders from several different species have demonstrated an important role for ROCK-induced calcium sensitization during contraction (Durlu-Kandilci and Brading, 2006; Fleichman et al., 2004; Schneider et al., 2004a; Takahashi et al., 2004; Wibberley et al., 2003). Several studies have also shown that PKC inhibition reduces carbachol-induced contractions in mouse, rat, guinea pig and human bladder tissue (Mizuno et al., 2008; Takahashi et al., 2004; Durlu-Kandilci and Brading, 2006). Thus the two main sensitizing pathways are present in the adult urinary bladder.

In addition to the regulation by myosin light chain phosphorylation, smooth muscle actin-myosin interaction can be modulated by thin filament associated proteins e.g. caldesmon and calponin. Their functions have not been completely elucidated in the organized contractile system. Caldesmon extraction or addition of caldesmon fragments in skinned muscle has been shown to affect Ca^{2+} sensitivity (Malmqvist et al., 1996; Pfitzer et al., 1993). Calponin has been suggested to be involved in the contractile kinetics and in regulation of shortening velocity without affecting force (Jaworowski et al., 1995; Matthew et al., 2000). Both caldesmon and calponin are also targets for kinases including PKC (Horowitz et al., 1996), which suggests that their properties can be modulated in response to agonist-induced activation.

Contractile system

The contractile apparatus of smooth muscle contains myosin (thick) and actin (thin) filaments. During contraction the cross-bridge projections from the myosin filaments interact in a cyclic manner with actin. In the urinary bladder, the relationship between muscle length and active force generation is broad and the smooth muscles can develop force over a wide length interval (van Mastrigt, 2002). In the human urinary bladder, active force is near maximal at cell lengths corresponding to filling volumes between 25-75% of maximal capacity (Uvelius and Gabella, 1980). The myosin forms a stable dimer that has two head regions and a tail region. Each head has ATPase activity and an actin-binding site (Eddinger and Meer, 2007). The contractile process

is coupled with hydrolysis of ATP to its products ADP and phosphate on the motor domain of myosin (Andersson and Arner, 2004).

The smooth muscle myosin molecule belongs to the myosin II family (Eddinger and Meer, 2007). Smooth muscle cells also express the vertebrate non-muscle myosins, non-muscle myosin A and non-muscle myosin B (Eddinger and Meer, 2007; Lofgren et al., 2003; Morano et al., 2000). These non-muscle myosins also form filaments and are thought to contribute to force generation similar to the smooth muscle myosin in some tissues although the contractile kinetics are slower and shortening velocity lower (Lofgren et al., 2003; Morano et al., 2000). The smooth muscle myosin molecule is composed of six polypeptides; two myosin heavy chains (approximately 200 kDa), two myosin essential light chains (17 kDa, LC_{17}) and two regulatory light chains (20 kDa, LC_{20}). Several isoforms of the smooth muscle heavy and light chains can be formed by alternative splicing and possibly affect contractile kinetics (Babu et al., 2000). The adult detrusor is a comparatively fast smooth muscle with expression of relatively high amounts of fast smooth muscle myosin isoforms.

Interstitial cells

The smooth muscle of the bladder wall interacts with several other cell types including nerves and urothelium. Interstitial cells of Cajal (ICC) are cells with a putative role in intestinal motility. Recently, cells resembling the ICC have been found in tissues of the lower urinary tract, particularly in the bladder. The role of interstitial cells (IC) in the bladder is not well understood but they have been proposed to be possible regulators of contractile activity (Brading and McCloskey, 2005; Lagou et al., 2006). ICs are suggested to interact with the urothelium and smooth muscles and to be involved in the generation and modulation of phasic activity. Increased numbers of interstitial cells have been obtained from patients with various pathologies including bladder overactivity (Brading and McCloskey, 2005).

Urinary incontinence and overactive bladder

Diseases involving the urinary bladder are very common and urinary incontinence is one of the most prevalent chronic diseases, affecting people at all ages (Wilson et al., 2001; Thom et al., 1997). In Sweden, 500 000 people suffer from urinary incontinence (SBU, 2000). The overactive bladder syndrome (OAB) is one of the major diseases of the lower urinary tract affecting millions of people in Europe with large socio-economical impact and with dramatic consequences for the affected individual (Klotz et al., 2007). The overactive bladder is defined as: “increased urinary urgency, with or without urge urinary incontinence, usually with frequency and nocturia” (Abrams et al., 2003). Detrusor overactivity is also the most common voiding dysfunction in children (Franco, 2007). The overactive bladder has specific contractile characteristics including increased spontaneous myogenic activity, altered responsiveness to stimuli and changes in smooth muscle structure (Chu and Dmochowski, 2006). The causes for the unwanted contractions in OAB are not fully understood and treatment options are limited and have only temporary effects (Milsom et al., 2000).

Developmental regulation of micturition and urinary bladder contractility

During development, an integrated control of the maturation of all components of bladder filling and emptying has to be achieved. The smooth muscles of the urinary bladder are present already at a fetal age of 10 weeks in humans and are morphologically different from the cell types found in the urethra (Gilpin and Gosling, 1983). At about the same time, acetylcholine-esterase positive nerves are visible in the wall of the urinary bladder (Gilpin and Gosling, 1983). The release of substance P from sensory nerves appears to develop at a later stage after birth (Maggi et al., 1988). In newborn animals, a spinal micturition reflex is activated from afferent fibers in the perigenital region triggered when the mother licks the newborn in this area (de Groat, 2002). During subsequent development, the animals gradually establish the adult voiding patterns. Interestingly, spinal cord injuries in adult animals cause a reemergence of the more primitive micturition reflex found in newborn (de Groat, 2002).

A key characteristic of urinary bladders from newborn and young animals is the appearance of coordinated spontaneous waves of calcium and associated spontaneous contractile activity (Kanai et al., 2007; Szell et al., 2003). This activity is potentiated by muscarinic agonists (Kanai et al., 2007). The mechanism involved in the increased contractile activity is not fully understood but local release of ACh has been proposed as an initiating factor (Kanai et al., 2007). It has also been suggested that the gradual down-regulation of spontaneous contractions during post-natal development is due to increased BK channel activity (Ng et al., 2007). These contractions may have a physiological function when the neural control mechanisms are immature. Some of these characteristics of the newborn bladder are similar to those of the overactive bladder.

In parallel with the development of the nervous control systems and the membrane properties, the contractile system and its regulatory components develop in the urinary bladder. At birth, non-muscle myosin is highly expressed in smooth muscle. Mice without smooth muscle myosin develop and can survive for a few days after birth with a contractile function in the bladder (Morano et al., 2000). Thus non-muscle myosin is a component, together with the smooth muscle myosin, in the bladder muscle contractile system in the neonatal period. Other contractile isoforms change from a fetal to an adult expression pattern, e.g. the smooth muscle myosin heavy chains (Lin et al., 2000; White et al., 1998). An embryonic form of the MLCK is expressed early during smooth muscle development and is gradually replaced by the adult form (Kamm and Stull, 2001). The MLCP exists in several isoforms and studies on developing chicken gizzard smooth muscle have revealed a developmental change in expression pattern which is correlated with a gradual down-regulation of cGMP-responsiveness and up-regulation of phosphatase activity (Khatri et al., 2001; Ogut and Brozovich, 2000).

At present very limited information is available regarding the contractile regulation in the newborn bladder. Although some properties of the newborn bladder contraction have been shown to differ from those of the adult, an integrated view of the cellular signaling, membrane receptor function and nervous control in the newborn bladder is not available. A characterization of the integrative contractile regulation is of importance for understanding smooth muscle development in general, the interaction

between the regulatory components in the cell, and the integrative processes involved in micturition. An increased knowledge of the physiology and the contractile regulation of the developing smooth muscles, where phasic contractile activity is prominent, could also provide information on the mechanisms of bladder overactivity in humans and avail novel principles for improved treatments of this disease.

AIMS

The aim of the thesis work was to investigate developmental changes in the contractile properties and cellular signaling of the mouse urinary bladder smooth muscle. We have examined urinary bladders from newborn mice, and compared them with adult bladders, to specifically address the following questions:

- Which are the main nerve-induced activation pathways in the newborn bladder?
- Are the receptor-mediated contractions similar in the bladder from adult and newborn mice?
- Are there differences in muscarinic receptor subtypes between bladders from adult and newborn mice?
- Which sources of calcium are utilized for contractile regulation?
- Is the calcium sensitivity of contraction different?
- Are there differences in MLCK and MLCP activities?
- Are calcium sensitization mechanisms operational in newborn bladders?
- Which are the contractile effects of the PKC and ROCK signaling pathways in newborn and adult bladders?

EXPERIMENTAL PROCEDURES

Animals

Urinary bladders from NMRI mice were used in all studies in this thesis. The newborn mice were 0-2 days old (weight ~1-2 g). The adult mice were 10-16 weeks old (20-30 g). In paper I, 3 week old mice (~13 g) were also used. This is the time point when the animals are weaned. The animals were euthanized and the urinary bladders were placed in cold physiological saline solution (PSS, composition is given in paper I). The urinary bladder was cut open through the urethra. The urothelium was left intact in the newborn bladders in all experiments, in order to minimize the handling of the thin preparations and to avoid possible damage to the muscular wall. The urothelium was removed from the adult bladders in paper I. Since contractions following muscarinic receptor activation (using carbachol) did not differ between adult bladder preparations with or without the urothelium, all experiments in papers II-IV were performed with intact urothelium. The experiments were performed according to European guidelines for animal research and approved by the local animal ethics committee.

Force measurements and electrical field stimulation

A major part of the experiments in this thesis involves *in vitro* force registrations, where the effects of different activators and inhibitors of contraction can be studied. Intact smooth muscle strips (~0.5 mm wide and 3-5 mm long) were dissected in the circular direction from the mid-portion of the bladder from newborn and adult mice and tied with silk tread at both ends. One end of the preparation was attached to a metal pin and the other to a force transducer in open organ baths in PSS (gassed with 95% O_2 and 5% CO_2, pH 7.4). The preparations were stretched to a passive tension (1-2 mN for newborn and 3-4 mN for adult strips) corresponding to optimal length for active force development. The preparations were equilibrated for a minimum of 30 minutes before different protocols were applied. The muscle strips were initially activated with high-K^+ to ensure optimal contractile function in the preparations after the dissection and mounting procedure. The specific protocols and drugs used in each paper are presented in Tables 1-3.

Nerve and receptor function

Electrical field stimulation (paper II) was used to induce contraction by activation of intramural nerves in the urinary bladder wall. It was carried out using platinum electrodes placed on both sides of the preparation in the organ bath. The tissue strips were first repeatedly activated at 20 Hz stimulation frequency (0.5 ms pulse duration and 5 seconds stimulation trains) at varying stimulation voltage. The optimal voltage for force development was used during the remaining part of the experiment. Frequency–response curves or repeated stimulation at a set frequency (20 Hz) were performed using 5 s train duration at 2-min intervals. To examine the contribution of cholinergic, adrenergic and purinergic transmitters, the preparations were treated with the muscarinic receptor inhibitor scopolamine, the α-adrenoceptor antagonist prazosin or the purinoceptor desensitizing compound α,β-methylene ATP (Table 1). The contribution of non-nerve-mediated activation to the electrical field stimulation-induced contractions was determined after inhibition of intramural nerves with tetrodotoxin (TTX). In principle, several frequency-response curves were recorded: an initial to determine the nerve responses at different frequencies and thereafter in the presence of the different inhibitors. In control experiments, five frequency-response relationships were recorded in the absence of drugs. As discussed in the Results/Discussion section, the responses of the adult bladder strips were reproducible whereas the responses of the newborn decayed with repeated stimulation. Therefore separate preparations were used for each compound in the frequency-response experiments in the newborn. In an alternative protocol the effects of the receptor inhibitors were examined using a fixed stimulation frequency (20 Hz).

To examine the contractile receptor function, EC_{50} values (i.e. the concentration giving half-maximal force) were determined for the muscarinic receptor agonist carbachol and for the purinoceptor agonists ATP and α,β-methylene ATP in adult and newborn tissues. The EC_{50} concentrations for carbachol in each tissue were used for examining ROCK activity and 8-Br-cGMP effects on muscarinic responses (paper II, cf. Table 2). In paper III, we examined the receptor subtypes involved in the cholinergic responses of the newborn and adult bladders. In these experiments we examined the responses to different doses of carbachol in the presence of the

muscarinic M_2 receptor antagonist methoctramine and the muscarinic M_3 receptor antagonist 4-DAMP (Choppin and Eglen, 2001; Eglen et al., 1994).

Table 1. Nerve and receptor function. Summary of the organ bath experiments used in the papers included in this thesis. CCh: carbachol, TTX: tetrodotoxin

Question	**Methodological approach**	**Paper**
Nerve function	Electrical field stimulation in the presence of scopolamine, prazosin, α,β–methylene ATP and TTX	II
Muscarinic receptor function	Concentration-response curves (EC_{50}) for CCh	II
Purinergic receptor function	Concentration-response curves (EC_{50}) for ATP and α,β–methylene ATP	II
Muscarinic receptor subtypes	Effects of methoctramine (M_2 receptor antagonist) and 4-DAMP (M_3 receptor antagonist) on CCh responses	III

Cellular signaling pathways

The cellular signaling pathways associated with Ca^{2+}-activation and receptor stimulation were studied in all papers in this thesis. The experiments are summarized in Table 2. As presented in the Introduction, the main activator of contraction is Ca^{2+}, which activates MLCK, which in its turn phosphorylates the regulatory myosin light chains. The measurements of intracellular [Ca^{2+}], the calcium sensitivity and the experiments involving influx and release of Ca^{2+} are described separately below. An important mode of activation is depolarization-induced opening of L-type Ca^{2+} channels. As an initial step we determined the optimal K^+-concentration ([K^+]) for activation of contraction (paper I) by activating the preparations at different extracellular [K^+]. Using this mode of activation, we examined the sensitivity to extracellular Ca^{2+} concentration and the MLCK activity (using an inhibitor of the kinase, ML-9 (Saitoh et al., 1987)). Non-muscle myosin has been suggested to be active in newborn bladders (Lofgren et al., 2003; Morano et al., 2000). To examine the relative contribution of this contractile protein, we used blebbistatin (Limouze et al., 2004; Straight et al., 2003) which has been suggested to inhibit non-muscle but not smooth muscle myosin. To examine the role of PKC and RhoA/ROCK in Ca^{2+} sensitization and activation pathways we used the PKC activator PDBu (phorbol 12,13-dibutyrate (Rasmussen et al., 1987)), the ROCK inhibitor Y-27632 (Uehata et al., 1997) and the PKC inhibitor GF109203X (Toullec et al., 1991). The role of

relaxant pathways dependent on nitric oxide (NO) was examined using the cell permeable cGMP analogue 8-Br-cGMP (Wu et al., 1996) which activates protein kinase G (PKG), L-NAME which inhibits NO synthesis (Persson et al., 1992) and ODQ (Young et al., 1996), which inhibits soluble guanylyl cyclase and cGMP generation. Muscles were activated with depolarization (high-K^+) and/or muscarinic receptor stimulation (carbachol).

In paper IV, where we focused on the responses dependent on PKC activity, we also used activation with prostanoids (FP receptor agonist $PGF_{2\alpha}$, EP receptor agonist PGE_2 and the TP receptor agonist U-46619) and ATP/α,β-methylene ATP. All inhibitors (and the PKC activator PDBu) were added >10 minutes before the contractile initiation.

Table 2. Cellular signaling. Summary of organ bath experiments. CCh: carbachol, PKC: protein kinase C, ROCK: Rho-kinase.

Question	**Methodological approach**	**Paper**
Depolarization-induced contractions	Concentration-response curves (EC_{50}) for KCl	I
MLCK activity at different [Ca^{2+}]	Calcium concentration-response and inhibition of MLCK with ML-9	I
Non-muscle myosin-induced contractions at different [Ca^{2+}]	Calcium concentration-response and inhibition of non-muscle myosin with blebbistatin	I
ROCK-induced contraction at different [Ca^{2+}]	Calcium concentration-response and inhibition of ROCK with Y-27632	I
ROCK activation in muscarinic receptor-induced responses	EC_{50} CCh and ROCK inhibition with Y-27632	II
PKC activity in muscarinic receptor-induced responses	CCh responses: activation of PKC with PDBu and inhibition of PKC with GF109203X	IV
PKC activity in purinergic receptor-induced responses	ATP/α,β-methylene ATP responses and activation of PKC with PDBu	IV
PKC activity in prostanoid receptor-induced responses	$PGF_{2\alpha}$, PGE_2 and U-46619 responses and activation of PKC with PDBu	IV
Relaxing pathways in muscarinic receptor-induced responses	EC_{50} CCh activation and 8-Br-cGMP-induced relaxation	II
Relaxing pathways in muscarinic receptor-induced responses	CCh responses and inhibition of nitric oxide synthase and guanylyl cyclase with L-NAME and ODQ, respectively	III

Function of membrane channels, sarcoplasmic reticulum and gap junctions

The role of different membrane ion channels, calcium release and gap junctions in the muscarinic receptor responses were studied in papers II, III and IV. The experiments are summarized in Table 3. In all these experiments, 1 µM of carbachol was used to induce contraction and the inhibitors were present at least 10 minutes before contractions were elicited. The L-type Ca^{2+} channels were inhibited with nifedipine and the T-type channels with $NiCl_2$ (Fry et al., 2006; Hagiwara et al., 1988). The Ca^{2+} release from the SR was investigated using two different compounds; ryanodine which inhibits the ryanodine receptor Ca^{2+}-release channel and thapsigargin which inhibits the Ca^{2+} reuptake (sarco/endoplasmic reticulum Ca^{2+} ATPase) into the SR (Rivera and Brading, 2006). Repeated stimulation with caffeine was used to empty the SR Ca^{2+} stores in the experiments with thapsigargin. The BK and SK channels were inhibited with the specific toxins charybdotoxin and apamin, respectively (Ledoux et al., 2008) and the gap junctional activity with 18-β-glycyrrhetinic acid (Hashitani et al., 2001).

Table 3. Channels and cellular communication in muscarinic receptor-induced responses. Summary of organ bath experiments. CCh: carbachol. BK: Big conductance K^+ channel, SK: Small conductance K^+ channel, SR: sarcoplasmic reticulum.

Question	**Methodological approach**	**Paper**
Role of L-type calcium channels	CCh responses and inhibition of L-type calcium channels with nifedipine	II
SR release of Ca^{2+}	CCh responses and inhibition of SR calcium release with ryanodine and thapsigargin	III
K^+ channel function	CCh responses and inhibition of BK channels and SK channels with charybdotoxin and apamin, respectively	III
Gap junctional transmission	CCh responses and inhibition of gap junction transmission with 18-β-glycyrrhetinic acid	III
Role of T-type calcium channels	CCh responses and inhibition of T-type calcium channels with $NiCl_2$	IV

Intracellular calcium measurements

Intracellular calcium concentration is the main regulator of contractile force and to examine if intracellular [Ca^{2+}] transients were altered in newborn bladders, we used the Fura-2 technique (Arner et al., 1998; Lucius et al., 1998). Fura-2 is a fluorescent compound for which the excitation spectrum depends on the calcium concentration. Fura-2 is introduced to the preparations as the acetoxymethyl ester (Fura-2/AM) and

Fura-2 is trapped inside the cells due to intracellular cleavage of the AM ester. When intracellular calcium concentration increases, the emitted light for excitation at 340 nm increases relative to light emission for excitation at 380 nm. Using a rotating filter wheel at the excitation side and detection of the emission at 510 nm, the ratio (R) between the emitted light at 340 and 380 nm excitations can be determined. The Fura-2 signal was calibrated in each preparation by determining R_{min} (the fluorescence ratio with 0 Ca^{2+}), R_{max} (the fluorescence ratio with 10 mM Ca^{2+}) and background fluorescence (quenching the Fura signal with 20 mM $MnCl_2$) in preparations permeabilized with ionomycin.

The calcium levels were calculated using an equation presented by Grynkiewicz (Grynkiewicz et al., 1985):

$$[Ca^{2+}]_i=(K_d \times I_{380F}/I_{380B}) \times (R-R_{min})/(R_{max}-R)$$

where $[Ca^{2+}]_i$ is the intracellular calcium concentration, K_d is the dissociation constant, I_{380F} is the fluorescence intensity at 380 nm excitation at minimal intracellular calcium, I_{380B} is the fluorescence intensity at 380 nm excitation at saturating intracellular calcium, R is the measured ratio between the fluorescence intensities at 340 and 380 nm excitation wavelengths.

In paper I, muscle preparations were loaded with Fura-2/AM in PSS for 3-4 hours at room temperature and then rinsed to wash out remaining Fura-2/AM in the tissue before mounting with aluminum clips in an open organ bath. Thereafter the preparations were stimulated with increasing concentrations of extracellular $CaCl_2$ in the depolarized (125 mM K^+) state. The intracellular calcium concentration and force were measured for 30 seconds at each extracellular concentration. From these experiments we determined the relations between extracellular $[Ca^{2+}]$, intracellular $[Ca^{2+}]$ and force in depolarized newborn and adult urinary bladders.

Permeabilized smooth muscle preparations

Chemical permeabilization of cell membrane in muscle preparations enables precise control of the intracellular environment in a system where the contractile apparatus is still operational. In Triton X-100 (Triton) treated preparations (used in paper I),

internal and external membranes are dissolved and regulatory mechanisms via receptor activation and cellular signaling are absent (e.g. PKC and ROCK). The contractile regulation in these preparations is mainly due to the activity of MLCK and MLCP, where the activity of the kinase is set by the Ca^{2+} and calmodulin concentrations. Urinary bladder strips were dissected and put in 1% Triton for 4 hours at 4ºC and then kept at -20ºC in a glycerol-containing solution until usage. Permeabilized smooth muscle preparations were mounted using aluminum foil clips in a solution containing 30 mM TES, 4 mM EGTA, 3.2 mM MgATP, phosphocreatine 12 mM and 2 mM free Mg^{2+} with an ionic strength of 150 mM. pH was adjusted to 6.9 using KOH. Since proteins are lost during Triton permeabilization, calmodulin and creatine kinase were added back in the solutions. To determine the calcium sensitivity (EC_{50} for calcium) the muscles were activated at increasing concentrations of free calcium, obtained by varying the CaEGTA/EGTA ratio in the solutions. To maximally activate the preparations thiophosphorylation of the light chains was used (Jaworowski et al., 1999). The preparations were pre-incubated with ATP-γ-S, calmodulin and Ca^{2+} for 10 minutes. This results in a near maximal thiophosphorylation of the light chains. The muscles were then contracted by transfer to an ATP containing solution. The maximal stress (force/area) was calculated from force and muscle dimensions in thiophosphorylated maximally activated Triton permeabilized preparations.

To confirm the results obtained with Triton permeabilized preparations (paper I), we used α–toxin permeabilization (5000-10000 units for 1 hour at room temperature with constant stirring, paper I). This is a gentler permeabilization method where the receptor signaling pathways (including small G-proteins and PKC) are left operational (Kitazawa et al., 1989). Force was recorded at intermediate (pCa 5.5) and maximal (pCa 4.5) [Ca^{2+}] using the same solutions as described above.

All experiments on permeabilized preparations were performed at room temperature.

Myosin light chain phosphatase and myosin light chain kinase activities

Since MLCK and MLCP are the main regulators of contraction in both intact and permeabilized tissue, we focused on the expression and activities of these proteins to understand differences in calcium sensitivity (paper I) between adult and newborn bladder tissue.

To estimate the rate of relaxation in newborn and adult tissue, we measured the half-time for relaxation in Ca^{2+}-free solution, after a maximal contraction in permeabilized preparations. In these experiments, tissue preparations were also quickly frozen at different time points during the relaxation and used for urea/glycerol gel electrophoresis (see below) and for determination of myosin light chain phosphorylation during the relaxation phase (see below). In another set of experiments the force decay during 1 hour was measured and used as an estimation of the MLCP activity. In these experiments, ATP-γ–S was used to (thio)phosphorylate the light chains. Thiophosphorylated light chains are weak substrates for MLCP and are therefore more slowly dephosphorylated. To confirm that the force decay was due to the activity of MLCP, control experiments were performed with the MLCP inhibitor microcystin LR.

The MLCK activity was estimated by measuring ATP-induced force development after increasing incubation time in ATP-γ-S-containing rigor solution at high [Ca^{2+}] (pCa 4.5). Since dephosphorylation of the thiophosphorylated light chains is near zero, the resulting force after activation with ATP can be used as an estimation of myosin light chain phosphorylation and thus MLCK activity.

Gel electrophoresis, Western blotting and myosin light chain phosphorylation

SDS-gel electrophoresis was performed in papers I, III and IV to determine the content of different proteins involved in the contractile regulation during development in the urinary bladder. Depending on the size of the proteins of interest, different percentages of polyacrylamide were used. The actin and myosin contents were determined in paper I after Coomassie blue staining using a standard with known concentration of skeletal muscle actin. Equal amounts of protein were loaded from the adult and newborn mice. In papers I, III and IV contents of specific proteins were investigated using Western blotting and specific antibodies (paper I: MYPT1 and

LC20, paper III: connexin 43 and paper IV: PKC). For Western blotting, the separated proteins were transferred to a nitrocellulose or PVDF membrane and the membrane was incubated with a primary antibody. The secondary antibody was labeled with HRP (horse radish peroxidase), which enables detection via a chemiluminescence signal.

Urea/glycerol gel electrophoresis is a method used for separating phosphorylated and unphosphorylated myosin light chains on the basis of charge. In paper I, myosin light chain phosphorylation was measured by urea/glycerol gel electrophoresis and Western blotting (Weber et al., 1999). Permeabilized preparations were stimulated at intermediate Ca^{2+} (pCa 5.8) and then quickly frozen and fixed in trichloroacetic acid containing 4 mM pyrophosphate. The preparations were washed with acetone and stored at -80ºC until subjected to electrophoresis and Western blotting as described above using an antibody against the regulatory myosin light chains. The unphosphorylated and the phosphorylated light chains have different migration lengths in the urea gels and can therefore be visualized as two different bands after Western blotting. The relative myosin light chain phosphorylation (phosphorylated light chains/total amount of light chains) was calculated.

To estimate the activity of the MLCP, myosin light chain phosphorylation was determined at different time points during relaxation in calcium free solution after a maximal contraction in pCa 4.5 (see above). The preparations were treated and subjected to urea/glycerol gel electrophoresis as described above and the relative myosin light chain phosphorylation at the different time points was calculated.

Histology

Histological sections provide information on structural differences in newborn and adult bladder tissue. Urinary bladders were fixed in 4% paraformaldehyde. The preparations were mounted with OCT and frozen in isopentane. Cryosections (10 µm) were stained with hematoxylin/eosin. The urothelium/lamina propria and the smooth muscle areas of the cross-sections were calculated from light microscopy images.

RESULTS AND DISCUSSION

Histology and contractile force

Newborn mice have a comparatively large urinary bladder with a 3-fold higher bladder/body weight ratio than adult animals (from paper III). Three main layers are found in the bladder wall of both newborn and adult mice: an outer thin serosa, a middle muscular layer and an inner layer consisting of urothelium and lamina propria (Figure 1 in paper III and Figure 4). These data are consistent with results from human bladders where these layers seem to be established very early during development and bundles of smooth muscles can be found already 10 weeks after gestation in humans (Gilpin and Gosling, 1983). It is possible that an interaction between the layers of the wall is important during growth. The epithelium of the bladder produces growth factors that influence smooth muscle development and differentiation (Liu et al., 2000). The smooth muscle layer is thinner in the newborn than in the adult, but it is still comparatively thick in relation to the total bladder wall thickness.

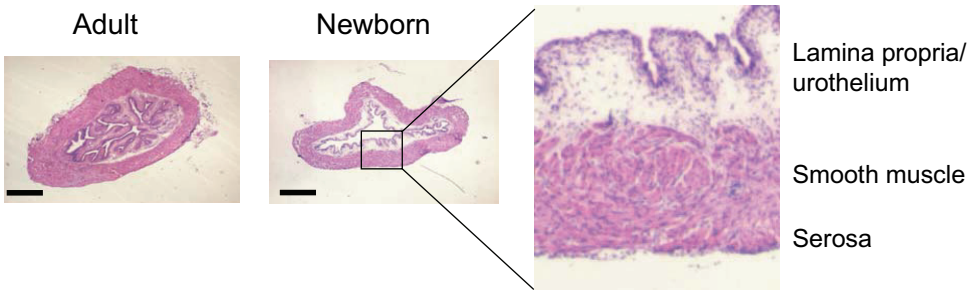

Figure 4. Histological sections of an adult and a newborn urinary bladder from mouse. The right section shows a magnification of the newborn bladder wall components. Bar = 0.5 mm. Modified from Paper III.

In paper I, we examined the maximal active force development of the smooth muscles in the urinary bladder. The preparations of the newborn included the thin urothelial layer since we wanted to avoid extensive manipulation of the thin wall of the newborn. We used permeabilized preparations and activation with irreversible thiophosphorylation using ATP-γ-S, to ensure maximal activation of the tissues. The maximal active wall stress, following thiophosphorylation of permeabilized

preparations, was lower in the newborn compared with that of the adult (in paper I; newborn 13.9 ± 1.8 and adult 21.5 ± 2.2 mN/mm^2). Part of the lower stress in the newborn could be explained by inclusion of urothelium/lamina propria in the newborn muscle preparations. These parts of the urinary bladder do not contribute to the active force generation. Quantification of actin and myosin proteins in the urinary bladder revealed a lower content of these proteins in the newborn, which, at least partly, could be explained by the presence of urothelium/lamina propria in the preparations used for protein determination. If we use the morphological data on urothelial area (30% of the total wall area in the newborn; Figure 1C in paper III) we can correct the stress and contractile protein contents in the newborn bladder. The value for force is ~90% and contents of myosin/actin are ~100% and ~85% of those in the adult after correction. These results show that even though the maximal force in the wall is slightly lower in newborn bladder, the smooth muscle contractile properties (i.e. force and contractile protein content per smooth muscle area) are fairly well established already at birth.

When the abdomen is opened in newborn animals, the urinary bladder is found to be distended and filled with urine. This distension disappears when the mice are about 3 days old. The cause of the distended bladder is not known. Since active force development is close to that of the adult bladders, the appearance of the filled bladder could be due to immature nervous regulation of micturition during the first days of extra uterine life.

Nerve- and receptor-mediated contractions

In paper II, we investigated developmental alterations in the nerve-mediated regulation of bladder contractility, using electrical field stimulation of intramural nerves and inhibitors of cholinergic, purinergic and adrenergic transmission. As mentioned in the Introduction, the adult responses were reproducible whereas the responses of the newborn decayed. This was not due to a general destruction of the newborn tissue since depolarization-induced contractions remained constant. It is possible that the nerves in the newborn bladders are more sensitive to high frequency stimulation or that the nerve transmitters are more easily depleted in the newborn. In the adult mouse bladder muscarinic and purinergic receptor activation are equally important for nerve-induced initiation of force (Figure 1 in paper II). In

contrast, the nerve-induced contractions of the newborn bladder are mainly mediated by cholinergic receptor activation, with a minor contribution of purinergic receptor activation (Figure 1 in paper II). To examine if the low purinergic receptor-induced responses in the newborn bladder were due to properties of the $P2X_1$ receptors, we examined the effects of ATP activation. When the purinergic receptors were activated directly (with ATP or α,β-methylene ATP), newborn and adult tissue responded in a similar way, which shows that differences in the nerve-mediated purinergic contractile regulation are due to differences upstream of the purinergic receptors (Figure 5 in paper II). Most likely the release of ATP from the nerve terminals or the nerve density of ATP releasing nerves is lower in the newborn. These results show that cholinergic signaling is the main activating pathway in newborn urinary bladders.

Muscarinic receptor-induced contractile responses

In paper II we observed that the carbachol-induced force development was different in newborn mice compared with adults (Figure 5). Bladders of newborn mice lack the characteristic plateau phase observed in bladders of older animals. Instead, the newborn bladders have short, phasic, high amplitude contractions during prolonged cholinergic stimulation superimposed on a very low sustained force (Figure 2 in paper III and Figure 5).

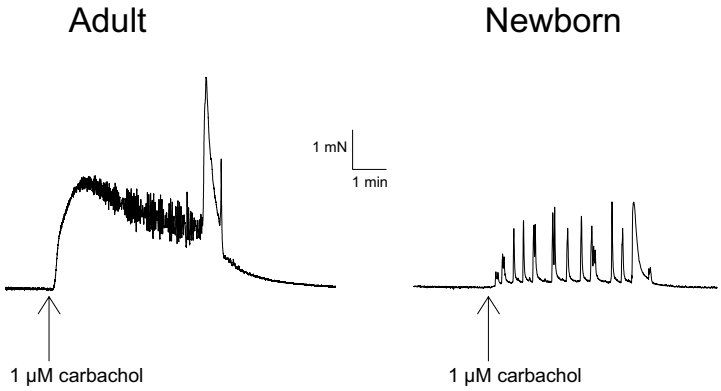

Figure 5. Original recordings of carbachol (1 µM) responses in adult and newborn bladder preparations. Modified from paper III.

To further characterize the muscarinic receptor-induced responses in the newborn, we elicited separate contractions with increasing concentrations of carbachol. When

evaluating these, we defined three different parameters of the contractile response: (i) the initial peak response obtained within 1 minute after addition of carbachol, (ii) the sustained force component 5 minutes after addition of carbachol and (iii) the amplitude of the short phasic contraction 5 minutes after addition of carbachol. The force value of these amplitudes was measured as the mean of three phasic contractions. The newborn bladders had a higher EC_{50} value for the peak response (i.e. a lower sensitivity) of carbachol compared with adults (Figure 4 in paper II). Possible explanations for this low sensitivity could be an altered receptor expression, changes in cellular signaling or lower capacity for receptor-induced calcium sensitization (as will be discussed below). In summary, newborn bladders have a different contractile response when activated with carbachol and a lower sensitivity to this agonist.

In adult animals, the muscarinic receptor subtype M_3 mediates the contractile response (Schneider et al., 2004b; Matsui et al., 2000) although M_2 receptors are dominant in number. The functional importance of different muscarinic receptor subtypes is not known in newborn animals and it is possible that a change in expression and function of these receptors is involved in the increased phasic activity of the urinary bladder of newborn mice. In paper III, we addressed this question using specific antagonists of M_2 (methoctramine) and M_3 (4-DAMP) receptors. The results showed that M_3 receptor activation is the main initiator of carbachol-induced force both in newborn and adult tissue, and thus the adult functional receptor pattern is established already in the newborn bladder (Figure 2 in paper III). The contribution of M_2 receptors was marginal in both the adult and the newborn. M_2 receptors have been shown to indirectly enhance contractility in the urinary bladder (Ehlert et al., 2005) and to inhibit MLCK in other smooth muscle tissues (Murthy et al., 2003). We could not find any major involvement of M_2 receptors in muscarinic receptor-induced activation of contraction. Experiments using carbachol in the presence of the M_3 antagonist in precontracted (with high-K^+) bladders did not reveal any relaxant responses in adult or newborn bladders (Figure 3 in paper III). This suggests that no relaxant M_2 receptor pathways are operating in the mouse bladder.

Intracellular calcium concentration is increased following muscarinic receptor activation. Ca^{2+} is recruited from either the extracellular environment via influx

through L-type calcium channels or from inside the cells via SR release (Andersson and Arner, 2004). In paper II, we show that the L-type Ca^{2+} channel inhibitor nifedipine abolished the carbachol responses of both newborn and adult bladders (Figure 6 in paper II). Thus influx via L-type calcium channels is the major source of activator calcium following muscarinic receptor activation in both the adult and newborn mouse urinary bladder. All phases of the contractile response (initial peak response, the sustained plateau phase and the amplitude of the short, phasic contractions) were affected in a similar manner by nifedipine in both adult and newborn bladder tissues. The importance of calcium release from SR seems to vary between species and due to experimental set-ups (Andersson and Arner, 2004; Rivera and Brading, 2006; Wuest et al., 2007). Our results (Figure 5 in paper III) show that the carbachol-induced responses in the adult and the newborn bladders are not dependent on calcium release from intracellular stores since the contractions were not affected by the sarcoplasmic calcium release inhibitor ryanodine or the calcium reuptake inhibitor thapsigargin. Cholinergic activation of the newborn and adult mouse bladder thus induces contraction mainly via activation of Ca^{2+} influx, with little or no contribution of Ca^{2+} from intracellular stores.

Release of calcium from SR has been shown to activate calcium dependent BK channels and induce hyperpolarization (Herrera et al., 2005). Although SR is not a major source of activator Ca^{2+}, this mechanism at the level of the cell membrane can be important for hyperpolarization and relaxation in the urinary bladder. In paper III we used inhibition of SR Ca^{2+} release and of BK channels. Both these interventions slightly potentiated carbachol-induced force in both adult and newborn urinary bladders. These results show that calcium regulated K^+ channels are functional already at birth in the mouse bladder (Figures 5 and 7 in paper III).

Spontaneous contractile activity of urinary bladders is known to change during development (Kanai et al., 2007; Szell et al., 2003). In young animals, this rhythmical activity is characterized by a high amplitude and low frequency (Kanai et al., 2007; Szell et al., 2003), which may promote voiding when the neural control is immature. Dysfunctional control of this activity might also lead to bladder instability later in life (Mills et al., 2000). The spontaneous activity in newborn animals is enhanced by carbachol (Kanai et al., 2007). Therefore we aimed at examining the cellular

mechanisms and origin of the phasic contractions in newborn bladders stimulated with carbachol (paper III). Spontaneous phasic activity of smooth muscle may involve many and complex mechanisms. A synchronization of the electrical activity of cells in combination with feedback loops initiating the oscillations could be important. Cell-cell coupling has been suggested to be involved in increased spontaneous activity of hypertrophied bladders (Christ et al., 2003). To examine if differences between gap junctional cell-cell communication can explain the difference in phasic activity between newborn and adult bladders, we used inhibitors of gap junctional activity and determined the content of the gap junction protein connexin 43 (Christ et al., 2003; Ikeda et al., 2007). No differences in connexin 43 expression could be detected between the newborn and adult tissues. Further, the gap junction inhibitor 18-β-glycyrrhetinic acid (and heptanol) did not inhibit the phasic activity of the carbachol activated newborn bladders in a different manner compared to the adult bladders (Figure 4 in paper III). These results show that differences in gap junctions/cell-cell communication are not responsible for the differences in phasic activity between adult and newborn mouse bladders. It should be noted that the electrical activity of bladder smooth muscle may be coordinated by other cell types, e.g. interstitial cells (Brading and McCloskey, 2005; Lagou et al., 2006), These cells are found under the lamina propria and are suggested to interact with the urothelium, the smooth-muscle bundles and with sensory nerves (Brading and McCloskey, 2005).

In other types of smooth muscles, phasic contractile activity has been shown to involve feedback loops with calcium release from SR (Peng et al., 2001). In vasomotion of blood vessels, a feedback loop involving SR Ca^{2+} release and Ca^{2+}/cGMP regulated Cl^- channels has been suggested (Nilsson and Aalkjaer, 2003). Calcium release from SR is also suggested to be involved in contractile regulation of spontaneous tone in blood vessels via activation of BK channels (Brenner et al., 2000). A similar mechanism has also been proposed in the urinary bladder. As presented in the Introduction, a feedback mechanism can be established involving Ca^{2+} release from the SR and opening of the Ca^{2+}-regulated BK/SK channels (Herrera et al., 2000). Thus an increase in intracellular [Ca^{2+}] can inhibit activity by hyperpolarization which then leads to closing of the L-type Ca^{2+} channels. To examine the role of a SR-BK/SK feedback mechanism in the generation of the phasic

activity in the M_3 stimulated newborn bladders, we inhibited the BK and SK channels with charybdotoxin and apamin, respectively, and found that phasic activity still was present (Figure 7 in paper III). Further, inhibition of the SR release with either thapsigargin or ryanodine did not influence the phasic responses (Figure 5 in paper III). Thus, the phasic activity following carbachol stimulation of the newborn does not involve an SR-K^+ channel feedback loop. A local release of nitric oxide could also be involved in the generation of phasic contractions. However, our experiments using L-NAME and ODQ suggest that neither NO nor cGMP in the tissue are required for the phasic responses (Figure 6 in paper III). Introducing 8-Br-cGMP (Figure 6 in paper II) resulted in a decreased force in a similar manner in both adult and newborn tissues. This shows that cGMP/PKG-mediated relaxation, possibly via lowering of [Ca^{2+}] or Ca^{2+} desensitization (Bonnevier et al., 2004), is operating in the newborn.

T-Type Ca^{2+} channels are implicated in the phasic activity of adult bladders (Fry et al., 2006; Yanai et al., 2006) and could thus provide a mechanism for phasic activity in newborns. These channels are activated at more negative membrane potentials than other channels and it is hypothesized that activation of these channels generates spontaneous activity and induces calcium influx that initiates activation of other channels (Fry et al., 2006). We used $NiCl_2$ which has been shown to inhibit T-type calcium channel currents (Hagiwara et al., 1988). It should be noted that $NiCl_2$ could also have inhibitory effects on other ion channels and exchangers. T-type Ca^{2+} channel inhibition reduced the muscarinic receptor-induced contractility of newborn bladders more than that of the adult bladders (Figure 6 in paper IV). Differential expression of this channel during smooth muscle development has not been reported. However, the $NiCl_2$ results suggest that T-type Ca^{2+} channel currents could be involved in the increased spontaneous activity in carbachol (and unstimulated) newborn bladders. Thus, carbachol activation of the newborn mouse bladder leads to fluctuations in intracellular Ca^{2+} with influx through both L- and T-type Ca^{2+} channels. The interplay between these channels and the further mechanisms underlying the oscillations remain to be determined.

Calcium sensitivity of contraction

Contraction of the urinary bladder detrusor smooth muscle is regulated by intracellular calcium levels and modulated by calcium sensitization pathways (Andersson and Arner, 2004). We have shown that the major source of activator calcium is the extracellular compartment (Figure 6 in paper II) whereas calcium release from SR does not contribute to contractions to a major extent (Figure 5 in paper III). The responses to activator Ca^{2+} can be modulated by several mechanisms: (i) altered MLCK/MLCP activities (ii) altered activities in regulatory systems influencing the kinase/phosphatase activities (e.g. ROCK and PKC) and (iii) phosphorylation independent mechanisms e.g. thin filament regulatory systems (e.g. caldesmon and calponin).

Intracellular calcium levels and calcium sensitivity were investigated in paper I. In the depolarized muscles, newborn urinary bladders had an increased calcium sensitivity of contraction, both to extracellular and intracellular calcium, compared with adult mice (Figure 6, modified from paper I). Each extracellular $[Ca^{2+}]$ resulted in a lower intracellular $[Ca^{2+}]$ in the newborn compared with the adult (Figure 6, modified from paper I). This increased Ca^{2+} sensitivity enables contractions at lower intracellular and extracellular $[Ca^{2+}]$ in the newborn bladder.

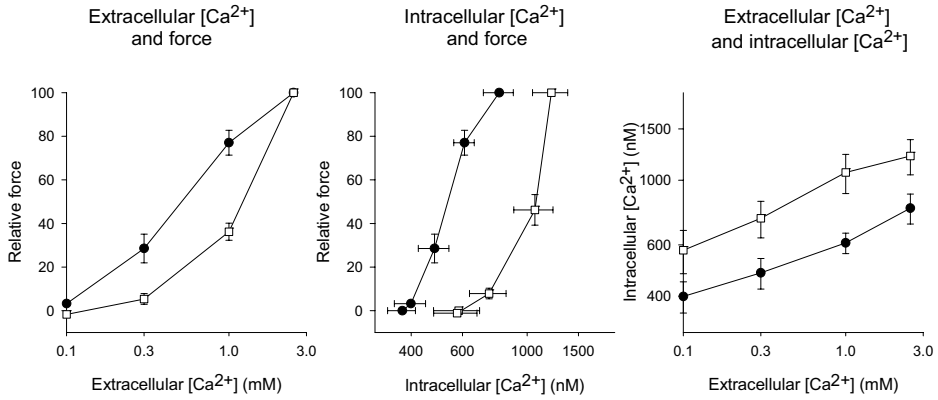

Figure 6. Force relative to extracellular and intracellular $[Ca^{2+}]$ and extracellular $[Ca^{2+}]$ relative to intracellular $[Ca^{2+}]$ in intact bladder preparations of newborn (filled circles) and adult (open squares). Modified from paper I.

The increased sensitivity to extracellular Ca^{2+} in the newborn mouse bladder was not due to higher levels of intracellular calcium (right panel of Figure 6). In a study by Zderic et al. (1991) on neonatal rabbit bladders, increased contractile responses to extracellular calcium and to cholinergic activation were associated with an increase in $[Ca^{2+}]_i$. This mechanism does not seem to be responsible for the increased sensitivity to extracellular Ca^{2+} in the newborn mouse bladder. The MLCK activity was not markedly different in newborn and adult bladder tissues (Figure 7 in paper I). Instead, we report that the increased calcium sensitivity is correlated with increased myosin light chain phosphorylation levels and a lower MLCP activity. The increased calcium sensitivity was shown both in intact tissue using determination of intracellular $[Ca^{2+}]$ (Figure 6) and in permeabilized bladder preparations at fixed $[Ca^{2+}]$ (Figures 5 and 6 in paper I). The permeabilization procedure with Triton removes the modulatory mechanisms, e.g. ROCK and PKC, and therefore calcium sensitization via these mechanisms cannot solely be responsible. Instead, we found differences in expression levels of the MLCP subunit MYPT1. The newborn tissue contained less than 50% of the MYPT1 in the adult tissue when related to the content of the regulatory light chains (Figure 8 in paper I). MYPT1 is the targeting subunit of the phosphatase that induces the co-localization of the phosphatase and the light chains and thereby determines the MLCP activity (Ito et al., 2004). A low MLCP activity was also demonstrated by determining the rate of dephosphorylation (Figure 7 in paper I) during relaxation. The low MLCP activity does not exclude that other signaling pathways upstream of MLCK/MLCP also contribute to the increased calcium sensitivity in the newborn mouse bladders. It is possible that PKC and ROCK activities are altered in the newborn or, since they terminate on the MYPT1, that their activities are affected by the altered phosphatase activity. MYPT1 can be alternatively spliced to generate 5 different isoforms in smooth muscle (Dirksen et al., 2000). The expression of these MYPT1 isoforms and the MLCP activity change during development in chicken smooth muscle (Khatri et al., 2001). It is possible that alternative splicing of MLCP subunits in the developing mouse bladder is involved in the increased calcium sensitivity. The isoform expression of MYPT1 also influences the cGMP/PKG-induced relaxation since they bind PKG differently (Khatri et al., 2001). We do not at present have data on the MYPT1 isoforms in the developing mouse bladder. It should be noted that PKG has several targets in the smooth muscle cell affecting calcium sensitization including ROCK (Sauzeau et al., 2000).

However, our observations that carbachol-induced contractions were present but less affected by 8-Br-cGMP in the newborn bladder (Figure 6 in paper II), are consistent with the view that the newborn bladders have developed the PKG targets and possibly express an MYPT1 isoform pattern with a lower responsiveness to PKG-induced relaxation.

Non-muscle myosins contribute to force development in newborn tissue including the newborn mouse bladder (Lofgren et al., 2003; Morano et al., 2000). The activation of non-muscle myosin is not known in detail but it is possible that the increased calcium sensitivity of the contractile system in the newborn is due to the content of non-muscle myosin. To address this issue we used blebbistatin (Limouze et al., 2004; Straight et al., 2003). Blebbistatin potently inhibits striated muscle myosins and non-muscle myosins whereas smooth muscle myosin is poorly inhibited (Limouze et al., 2004). Blebbistatin inhibited 50% of force generation in the newborn bladder but did not affect the contraction of adult (Figure 4 in paper I). These results are consistent with a relatively higher content of non-muscle myosin in the newborn bladder. The Ca^{2+} sensitivity of contraction was not influenced by blebbistatin (Figure 4 in paper I), which shows that the non-muscle component in the newborn bladder wall is not responsible for the increased Ca^{2+} sensitivity.

The cGMP/PKG pathway has not been studied in detail in newborn smooth muscle and it is possible that the lower content of MYPT1 alters the importance of this pathway in the newborn bladder. We report that 8-Br-cGMP inhibited carbachol-induced force in both adult and newborn (Figure 6 in paper II) which suggests that calcium desensitization pathways (via PKG) are operational in both tissues. The effect of 8-Br-cGMP on the sustained force was however lower in newborn tissue which could be due to a lower state of preceding calcium sensitization, a difference in PKG activity or in the PKG targets. In paper III we show, using L-NAME, that NO signaling is not involved in the muscarinic receptor-induced responses (Figure 6 in paper III). Thus although cGMP-mediated relaxant pathways can affect the carbachol activated bladder tissues from both adult and newborn animals, the upstream activator nitric oxide is not active in the carbachol-induced contractions.

Rho-kinase and protein kinase C

In addition to variation in intracellular [Ca^{2+}], force can be modulated by the activation and deactivation of ROCK and PKC (Somlyo and Somlyo, 2003). These kinases influence the Ca^{2+} sensitivity of the contractile system via inhibition of MLCP. The activities of the two main Ca^{2+}-sensitization pathways were investigated in papers I, II and IV.

The contribution of ROCK was examined in papers I and II. We show that ROCK activity is important for contraction induced by both depolarization and muscarinic receptor activation in newborn mouse bladders (Figure 4 in paper I and Figure 6 in paper II). In the newborn, the sustained component of the carbachol-induced contractions was less affected by ROCK inhibition compared with that of the adult and the phasic contractions were unaffected by ROCK inhibition (Figure 6 in paper II). This suggests that calcium sensitization mechanisms, via activation of ROCK, are contributing to force but not involved in the phasic contractions in the newborn. The lower extent of ROCK-mediated calcium sensitization on the plateau force in the newborn could be due to lower expression of the ROCK target MLCP (found in paper I) or to differences in RhoA/ROCK proteins.

In paper IV, we investigated the role of PKC on urinary bladder contractility. Using Western blots we showed that the main bladder smooth muscle PKC isoform (PKCα, (Persson et al., 1995)) was expressed in similar amounts in newborn and adult mouse bladders (Figure 7, and Figure 1 in paper IV). In a first set of experiments, we studied the effects of PDBu-induced activation of PKC on carbachol responses. In the adult bladder, carbachol-induced force was potentiated by PDBu (Figure 1 in paper IV, and Figure 7) which is consistent with a previous report (Lin et al., 1998). PKC can have several targets in the cell (Somlyo and Somlyo, 2003) and some of them affect Ca^{2+} handling (Lin et al., 1998; Park et al., 2006). The potentiation in the adult bladders can also involve an increased Ca^{2+} sensitivity via PKC dependent phosphorylation of the protein CPI-17 which inhibits the activity of the MLCP. A novel and interesting finding was that PDBu-induced activation of PKC abolished the carbachol responses in the newborn (Figure 1 in paper IV, and Figure 7).

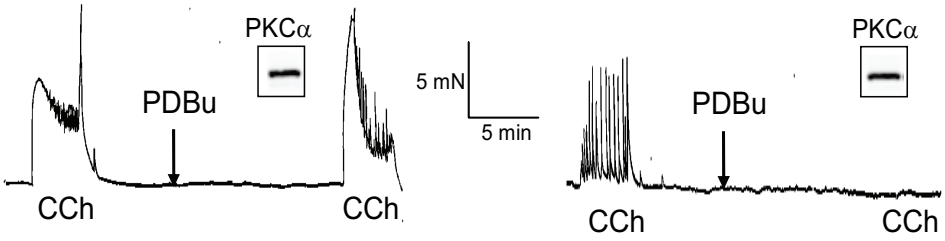

Figure 7. Original recordings of carbachol induced responses after incubation with PDBu in adult (left) and newborn (right) bladder tissue. Insets show Western blots of PKCα. From paper IV.

We performed experiments with the PKC inhibitor GF109203X to confirm that the effects of PDBu were due to specific activation of PKC. The experiments showed that the inhibitory effect of PDBu in the newborn could be reversed by addition of GF109203X (Figure 1 in paper IV). In another set of experiments, without preceding PDBu incubation, we showed that carbachol-induced force was attenuated by GF109203X (Figure 2 in paper IV). Peak force was inhibited by 60% and 30% in newborn and adult tissue, respectively, showing that PKC activity is contributing to the carbachol responses. It is notable is that the high amplitude phasic contractions in the newborn were unaffected by GF109203X, implying that PKC activity is not necessary for the phasic contractile activity of newborn bladders (Figure 2 in paper IV). The potentiating effects of PDBu in 40 mM K^+ depolarized tissue were similar in newborn and adult tissue (Figure 3 in paper IV), showing that PKC-mediated calcium sensitization is involved in depolarized-induced contractile responses in both tissues.

To examine if the phasic contractions observed after carbachol-induced activation in the newborn bladder also were present in other modes of receptor activation we examined the responses to prostanoids. The contractile responses to $PGF_{2\alpha}$ and PGE_2 were very small in both adult and newborn bladders. However, activation of thromboxane receptors with U-46619 resulted in prominent phasic contractions in the newborn, showing that the phasic activity response is not unique to the muscarinic M_3 receptors, but depend on receptor coupled signaling. Interestingly, the U-46619 contractions were inhibited by PDBu in a similar manner to the phasic contractions induced by muscarinic receptor activation. In arteries, it has been suggested that activation of thromboxane receptors can inhibit K^+ channels via PKC activation and

thereby depolarize the membrane triggering calcium influx via L-type calcium channels (Cogolludo et al., 2003). If this mechanism is operational in U-46619 stimulated mouse bladders, and whether it is responsible for the phasic responses or the PDBu inactivation in the newborn bladder is not known.

PKCα was expressed to a similar extent in newborn and adult bladders. We did not examine the expression of other PKC isoforms. Therefore it can not be excluded that the PDBu-dependent PKC inhibition of carbachol responses in the newborn is in part due to a different PKC isoform composition or a difference in PDBu-induced modification of PKC activity (Persson et al., 1995). An alternative possibility to altered expression of PKC isoforms in the newborn bladder is an alteration in the PKC targets. It is possible that high PKC activity inhibits the coupling between receptor and cellular activation in the newborn by phosphorylating an as yet unidentified target. One possibility is PKC dependent activation of G-protein coupled receptor kinases. Activation of these kinases would induce internalization of the M_3 receptor (Kelly et al., 2008; Lorenz et al., 2003; van Koppen and Kaiser, 2003). PKC is also suggested to phosphorylate T-type calcium channels (Park et al., 2006), thereby modulating the activity of this channel.

The results of paper IV thus clearly show that carbachol activation of both newborn and adult bladders recruits a PKC-mediated pathway potentiating the contraction (Figure 2 in paper IV), most likely via Ca^{2+} sensitization or membrane effects. In addition, the ROCK pathway is also activated (papers I and II). It appears these pathways are already functional in the mouse bladder at birth. The PKC pathway appears to be slightly more prominent and the ROCK slightly less prominent in the newborn when compared with the adult bladders. In addition to these effects of PKC and ROCK, a novel inhibitory mechanism appears to be operating at high levels of PKC activation in the newborn bladder. This mechanism directly affects the coupling between receptor activation and the contractile response.

SUMMARY AND CONCLUSIONS

Contraction of the urinary bladders of newborn mice is initiated by the activation of muscarinic M_3 receptors via ACh release from its cholinergic nerves. The purinergic component of the nerve response is small. The extracellular compartment is the main source of activator calcium through L-type calcium channels, whereas the SR contribution seems to be minor. The contractile responses to muscarinic receptor activation in the newborn are characterized by a prominent phasic activity. This phasic activity is not dependent on calcium release from SR, activation of BK or SK channels or gap junctional transmission. T-type calcium channels are suggested to be involved in the generation of phasic contractions in the newborn carbachol responses. Calcium sensitivity, both to extracellular and intracellular [Ca^{2+}], is increased in newborn mouse bladder when activated with high-K^+. The increased calcium sensitivity is correlated with a lower rate of dephosphorylation of the regulatory myosin light chains and a lower expression of the phosphatase subunit MYPT1, suggesting that altered expression of the phosphatase is responsible for modulation of calcium sensitivity during development. Muscarinic receptor activation and depolarization-induced contraction are influenced by calcium sensitization via activation of ROCK and PKC. Thus the main activation pathway (muscarinic receptor subtype M_3) and the major sensitization pathways (ROCK and PKC) are already established in the urinary bladder at birth. High level of PKC activation induces a specific inhibition of receptor-induced responses in the newborn, which can reflect either altered PKC isoform expression or unique inhibitory PKC targets in the receptor-contraction coupling in the newborn.

The contraction of the newborn bladder thus exhibits most general aspects of the adult bladder with regard to cellular signaling and receptor properties. The activation is shifted toward a situation with a low Ca^{2+} influx and a high Ca^{2+} sensitivity. This could reflect a developmental alteration in the L-type Ca^{2+} channel expression with a compensatory change in the contractile activation system. It is possible a resetting of the intracellular Ca^{2+} levels can be important for coordinating the contractile activity with other cellular Ca^{2+}-regulated processes. These cellular properties in the developing bladder are most likely also important for adapting the contractile responses to the extracellular control systems. The increased phasic activity of

newborn may be necessary for initiation of micturition when the nervous control system is not completely established. In some aspects, newborn bladders exhibit properties that are similar to overactive bladders, including increased spontaneous myogenic tone. The mechanisms involved in regulation of phasic contractile activity in the newborn, including the role of T-type channels and PKC inhibition may provide novel targets for inhibiting spontaneous tone in the overactive bladder condition.

SVENSK SAMMANFATTNING

Sjukdomar som drabbar urinblåsan är vanliga och berör cirka en halv miljon svenskar. Som exempel kan nämnas inkontinens och överaktiv blåsa. Detta är mycket besvärande tillstånd för dem som drabbas och samtidigt kostsamma sjukdomar för samhället. Vi föds med en fungerande urinblåsa även om spädbarn inte helt kan kontrollera tömningen av urinen. Hur urinblåsan utvecklas och anpassar sig under den första tiden efter födelsen är inte helt utrett. Denna process innefattar ett samspel mellan urinblåsans tillväxt och utveckling av dess nerver och muskulatur. I avhandlingen studeras funktionen i den nyfödda urinblåsan för att belysa dessa utvecklingsaspekter. Samtidigt kan denna information om urinblåsans funktion och styrning belysa egenskaper hos urinblåsan vid olika sjukdomstillstånd.

Urinblåsans muskulatur består av s.k. glatta muskelceller som regleras utan viljans kontroll. Vad som styr dessa cellers förmåga att dra ihop sig, kontrahera, eller slappna av, relaxera, är inte helt klarlagt. I avhandlingen undersöks de faktorer som påverkar kontraktionen av de glatta muskelcellerna i urinblåsan och hur dessa faktorer förändras under den första tiden efter födelsen.

När urinblåsan ska tömmas sänder nerverna ut ämnen (nervtransmittorer) som påverkar de glatta muskelcellerna som drar ihop sig och trycker ut urinen. Dessa transmittorer binder till, och aktiverar, olika receptorer på muskelcellernas yta. Inne i den glatta muskelcellen finns två kontraktila proteiner, aktin och myosin, som samarbetar för att muskelcellen ska utveckla kraft och förkortas. Kontraktionen i urinblåsan styrs av kalciumnivåerna inne i muskelcellen och av enzym som fosforylerar (myosin light chain kinase) eller defosforylerar (myosin light chain phosphatase) myosinet. Fosforylering av myosinet leder till aktivering och kraftgenerering medan defosforylering deaktiverar myosinet och kraften minskar. Andra faktorer som påverkar blåsan kontraktion är aktivering av olika s.k. cellsignaleringsvägar. De två viktigaste cellsignaleringsvägarna i glatt muskel involverar proteinkinas C och Rho kinas.

I de arbeten som ingår i avhandlingen studeras urinblåsor från möss för att undersöka grundläggande fysiologiska regleringsmekanismer i detta organ under

nyföddhetsperioden. Vi har visat att nyfödda urinblåsor aktiveras via s.k. muskarina receptorer (arbete II) och att aktivering av dessa receptorer leder till en kontraktion med en ökad oscillation av kraften (arbete II) jämfört med äldre djur som har en stabilare kontraktion. I den vuxna blåsan initieras kontraktionen via aktivering av både muskarina och s.k. purinerga receptorer. Av de muskarina receptorerna är det M_3 populationen som är viktigast för kontraktionsinitiering i både nyfödd och vuxen blåsa (arbete III). Den nyfödda blåsan är mer känslig för kalcium än den vuxna, vilket möjliggör kontraktion vid lägre kalciumnivåer både inne i cellen och utanför (arbete I). Detta beror på en lägre aktivitet och mängd av fosfataset (myosin light chain phosphatase) (arbete I). I den nyfödda vävnaden är även en speciell form av myosin (non-muscle myosin) viktig för kontraktionen, men aktivering av denna myosinform kan inte förklara den ökade kalciumkänsligheten (arbete I) hos de nyfödda. Vid aktivering av muskarina receptorer rekryteras kalcium från utsidan av cellen, via inflöde genom s.k. L-typ kalciumkanaler, i både nyfödda och vuxna blåsor (arbete II). I den nyfödda blåsan har även s.k. T-typ kalciumkanaler en betydelse för kontraktionsregleringen och aktivering av dessa kan delvis förklara den ökade oscillationen i kraften hos de nyfödda. De viktigaste cellsignaleringsvägarna, aktivering av proteinkinas C och Rho kinas, är etablerade i urinblåsan redan vid födseln (arbete I, II och IV). Vid hög aktivering av proteinkinas C kan inte muskarin aktivering inducera kraft hos den nyfödda blåsan (arbete IV), vilket skiljer sig från den vuxna där kraftutvecklingen ökar efter aktivering av proteinkinas C.

Sammanfattningsvis visar studierna i avhandlingen att kontraktionen av nyfödda blåsor är mer beroende av aktivering av muskarina receptorer, har en mer fasisk kraftutveckling, har en förändrad kalciumkänslighet och svarar annorlunda på proteinkinas C-aktivering jämfört med den vuxna blåsan. Dessa faktorer kan vara av betydelse för att möjliggöra tömning när blåsans yttre kontrollsystem inte är fullt utvecklade. Det kontraktila svaret vid muskarin aktivering hos nyfödda blåsor liknar den situation som finns vid överaktiv blåsa och kan eventuellt fungera som modell för att studera mekanismer som ger upphov till oönskad fasisk aktivering av urinblåsan hos människan. Fynden i denna avhandling bidrar till förståelsen av normal fysiologisk utveckling och reglering av glatt muskulatur i urinblåsan och denna kunskap kan även bidra till ökad förståelse för uppkomstmekanismer bakom sjukdomar som drabbar urinblåsan hos människor.

ACKNOWLEDGEMENTS

I would like to express my gratitude to everyone who has contributed in some way to this thesis.

Anders Arner, my supervisor, who introduced me to the field of smooth muscle physiology, for your guidance and your never ending enthusiasm.

All colleagues in the group of Vascular Physiology in Lund: Karl Swärd, for always having time for advice and support and for sharing your enthusiasm for science. Ina Nordström, for helping me out in the lab with all kinds of things. Per Hellstrand, for giving good advice and letting me be part of your lab. Bengt-Olof Nilsson, for your good mood and support. Kristina Andersson and Yulia Shakirova for being good friends and sharing hard times as well as good ones. Awahan Rahman, my room mate whose company I really enjoyed. Jingli, Anders, Kaj, Daniel…… for contributing to the great lab atmosphere. Former members of the group: Maria Gomez, Lisa Nilsson, Jenny Nilsson Öhman and Sebastian Albinsson, for inspiration and guidance in science.

People at Clinical Pharmacology in Lund: Karl-Erik Andersson, for great scientific input. Brita Sundén-Andersson, Agneta Kristensen and Christina Falk Olsson, for helping me out with technical things and for making me feel welcome in the lab. Ulf Malmqvist, for interesting discussions and good suggestions.

Former colleagues at Fysiologen in Lund: Johanna Balogh, a true friend and a great colleague. You made me survive the first years of scientific “depression”. Thank you a lot. Johan Bonnevier, for your inspiration and sparring in both science and in sports. My co-authors: Mia Wede, Katarina Fagher and Karolina Stakeberg, for good collaboration. Paul Edman, for sharing your impressive scientific knowledge.

The group of Genetic Physiology at Karolinska Institutet for making my visits to Stockholm enjoyable and full of coffee breaks and nice dinners, special thanks to Ben Davis and Piet Boels, for critically reviewing this thesis.

All present and past colleagues at Fysiologen and BMC D12, it has been a pleasure to work with you.

Friends and family:

Vänner, ingen nämnd ingen glömd. Livet skulle verkligen vara trist utan er.
Familjen Bech, tack för all hjälp med allt ifrån barnpassning till tältresning.
Familjen Grinneland, ni har lärt mig mycket och Mårten, tack för du alltid tror på mig.
Mamma och pappa: ert stöd gör det mesta möjligt. Tack.
Min familj: Ronnie, Melker och Malte, ni utmanar mig i allt. Tack för att ni finns.

This work was supported by The Research School in Pharmaceutical Science, FLÄK.

REFERENCES

Abrams,P. 2003. Describing bladder storage function: overactive bladder syndrome and detrusor overactivity. *Urology* 62:28-37.

Abrams,P., K.E.Andersson, J.J.Buccafusco, C.Chapple, W.C.de Groat, A.D.Fryer, G.Kay, A.Laties, N.M.Nathanson, P.J.Pasricha, and A.J.Wein. 2006. Muscarinic receptors: their distribution and function in body systems, and the implications for treating overactive bladder. *Br. J. Pharmacol.* 148:565-578.

Abrams,P., L.Cardozo, M.Fall, D.Griffiths, P.Rosier, U.Ulmsten, P.Van Kerrebroeck, A.Victor, and A.Wein. 2003. The standardisation of terminology in lower urinary tract function: report from the standardisation sub-committee of the International Continence Society. *Urology* 61:37-49.

An,J.Y., H.S.Yun, Y.P.Lee, S.J.Yang, J.O.Shim, J.H.Jeong, C.Y.Shin, J.H.Kim, D.S.Kim, and U.D.Sohn. 2002. The intracellular pathway of the acetylcholine-induced contraction in cat detrusor muscle cells. *Br. J. Pharmacol.* 137:1001-1010.

Andersson,K.E. 1993. Pharmacology of lower urinary tract smooth muscles and penile erectile tissues. *Pharmacol. Rev.* 45:253-308.

Andersson,K.E. and A.Arner. 2004. Urinary bladder contraction and relaxation: physiology and pathophysiology. *Physiol Rev.* 84:935-986.

Andersson,K.E. and P.Hedlund. 2002. Pharmacologic perspective on the physiology of the lower urinary tract. *Urology* 60:13-20.

Arner,A., U.Malmqvist, and R.Rigler. 1998. Calcium transients and the effect of a photolytically released calcium chelator during electrically induced contractions in rabbit rectococcygeus smooth muscle. *Biophys. J.* 75:1895-1903.

Babu,G.J., D.M.Warshaw, and M.Periasamy. 2000. Smooth muscle myosin heavy chain isoforms and their role in muscle physiology. *Microsc. Res. Tech.* 50:532-540.

Bonnevier,J., R.Fassler, A.P.Somlyo, A.V.Somlyo, and A.Arner. 2004. Modulation of Ca2+ sensitivity by cyclic nucleotides in smooth muscle from protein kinase G-deficient mice. *J. Biol. Chem.* 279:5146-5151.

Brading,A.F. and K.D.McCloskey. 2005. Mechanisms of Disease: specialized interstitial cells of the urinary tract--an assessment of current knowledge. *Nat. Clin. Pract. Urol.* 2:546-554.

Brenner,R., G.J.Perez, A.D.Bonev, D.M.Eckman, J.C.Kosek, S.W.Wiler, A.J.Patterson, M.T.Nelson, and R.W.Aldrich. 2000. Vasoregulation by the beta1 subunit of the calcium-activated potassium channel. *Nature* 407:870-876.

Caulfield,M.P. and N.J.Birdsall. 1998. International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol. Rev.* 50:279-290.

Choppin,A. and R.M.Eglen. 2001. Pharmacological characterization of muscarinic receptors in mouse isolated urinary bladder smooth muscle. *Br. J. Pharmacol.* 133:1035-1040.

Christ,G.J., N.S.Day, M.Day, W.Zhao, K.Persson, R.K.Pandita, and K.E.Andersson. 2003. Increased connexin43-mediated intercellular communication in a rat model of bladder overactivity in vivo. *Am. J. Physiol Regul. Integr. Comp Physiol* 284:R1241-R1248.

Chu,F.M. and R.Dmochowski. 2006. Pathophysiology of overactive bladder. *Am. J. Med.* 119:3-8.

Cogolludo,A., L.Moreno, L.Bosca, J.Tamargo, and F.Perez-Vizcaino. 2003. Thromboxane A2-induced inhibition of voltage-gated K+ channels and pulmonary vasoconstriction: role of protein kinase Czeta. *Circ. Res.* 93:656-663.

de Groat,W.C. 2006. Integrative control of the lower urinary tract: preclinical perspective. *Br. J. Pharmacol.* 147 Suppl 2:S25-S40.

de Groat,W.C. 2002. Plasticity of bladder reflex pathways during postnatal development. *Physiol Behav.* 77:689-692.

Dirksen,W.P., F.Vladic, and S.A.Fisher. 2000. A myosin phosphatase targeting subunit isoform transition defines a smooth muscle developmental phenotypic switch. *Am. J. Physiol Cell Physiol* 278:C589-C600.

Dixon J and J.A.Gosling. 1990. Ultrastructure of smooth muscle cells in the urinary system. *In* Ultrastructure of Smooth Muscle. Motta PM, editor. Kluwer Academic, London. 153-169.

Durlu-Kandilci,N.T. and A.F.Brading. 2006. Involvement of Rho kinase and protein kinase C in carbachol-induced calcium sensitization in beta-escin skinned rat and guinea-pig bladders. *Br. J. Pharmacol.* 148:376-384.

Eddinger,T.J. and D.P.Meer. 2007. Myosin II isoforms in smooth muscle: heterogeneity and function. *Am. J. Physiol Cell Physiol* 293:C493-C508.

Eglen,R.M., S.S.Hegde, and N.Watson. 1996. Muscarinic receptor subtypes and smooth muscle function. *Pharmacol. Rev.* 48:531-565.

Eglen,R.M., H.Reddy, N.Watson, and R.A.Challiss. 1994. Muscarinic acetylcholine receptor subtypes in smooth muscle. *Trends Pharmacol. Sci.* 15:114-119.

Ehlert,F.J., M.T.Griffin, D.M.Abe, T.H.Vo, M.M.Taketo, T.Manabe, and M.Matsui. 2005. The M2 muscarinic receptor mediates contraction through indirect mechanisms in mouse urinary bladder. *J. Pharmacol. Exp. Ther.* 313:368-378.

Fleichman,M., T.Schneider, C.Fetscher, and M.C.Michel. 2004. Signal transduction underlying carbachol-induced contraction of rat urinary bladder. II. Protein kinases. *J. Pharmacol. Exp. Ther.* 308:54-58.

Franco,I. 2007. Overactive bladder in children. Part 1: Pathophysiology. *J. Urol.* 178:761-768.

Fry,C.H., G.Sui, and C.Wu. 2006. T-type Ca2+ channels in non-vascular smooth muscles. *Cell Calcium* 40:231-239.

Gabella,G. and B.Uvelius. 1990. Urinary bladder of rat: fine structure of normal and hypertrophic musculature. *Cell Tissue Res.* 262:67-79.

Gilpin,S.A. and J.A.Gosling. 1983. Smooth muscle in the wall of the developing human urinary bladder and urethra. *J. Anat.* 137 (Pt 3):503-512.

Grynkiewicz,G., M.Poenie, and R.Y.Tsien. 1985. A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440-3450.

Hagiwara,N., H.Irisawa, and M.Kameyama. 1988. Contribution of two types of calcium currents to the pacemaker potentials of rabbit sino-atrial node cells. *J. Physiol* 395:233-253.

Hashitani,H., H.Fukuta, H.Takano, M.F.Klemm, and H.Suzuki. 2001. Origin and propagation of spontaneous excitation in smooth muscle of the guinea-pig urinary bladder. *J. Physiol* 530:273-286.

Herrera,G.M., B.Etherton, B.Nausch, and M.T.Nelson. 2005. Negative feedback regulation of nerve-mediated contractions by KCa channels in mouse urinary bladder smooth muscle. *Am. J. Physiol Regul. Integr. Comp Physiol* 289:R402-R409.

Herrera,G.M., T.J.Heppner, and M.T.Nelson. 2000. Regulation of urinary bladder smooth muscle contractions by ryanodine receptors and BK and SK channels. *Am. J. Physiol Regul. Integr. Comp Physiol* 279:R60-R68.

Herrera,G.M. and M.T.Nelson. 2002. Differential regulation of SK and BK channels by Ca(2+) signals from Ca(2+) channels and ryanodine receptors in guinea-pig urinary bladder myocytes. *J. Physiol* 541:483-492.

Horowitz,A., C.B.Menice, R.Laporte, and K.G.Morgan. 1996. Mechanisms of smooth muscle contraction. *Physiol Rev.* 76:967-1003.

Ikeda,Y., C.Fry, F.Hayashi, D.Stolz, D.Griffiths, and A.Kanai. 2007. Role of gap junctions in spontaneous activity of the rat bladder. *Am. J. Physiol Renal Physiol* 293:F1018-F1025.

Ito,M., T.Nakano, F.Erdodi, and D.J.Hartshorne. 2004. Myosin phosphatase: structure, regulation and function. *Mol. Cell Biochem.* 259:197-209.

Jaworowski,A., K.I.Anderson, A.Arner, M.Engstrom, M.Gimona, P.Strasser, and J.V.Small. 1995. Calponin reduces shortening velocity in skinned taenia coli smooth muscle fibres. *FEBS Lett.* 365:167-171.

Jaworowski,A., N.Ozturk, and A.Arner. 1999. Inhibition of force and shortening in smooth muscle by vanadate. *Pflugers Arch.* 438:224-231.

Jiang,H.H., B.Song, G.S.Lu, Q.J.Wen, and X.Y.Jin. 2005. Loss of ryanodine receptor calcium-release channel expression associated with overactive urinary bladder smooth muscle contractions in a detrusor instability model. *BJU. Int.* 96:428-433.

John,H., D.Hauri, U.Bangerter, and A.Elbadawi. 2001. Ultrastructure of the trigone and its functional implications. *Urol. Int.* 67:264-271.

Kamm,K.E. and J.T.Stull. 2001. Dedicated myosin light chain kinases with diverse cellular functions. *J. Biol. Chem.* 276:4527-4530.

Kanai,A., J.Roppolo, Y.Ikeda, I.Zabbarova, C.Tai, L.Birder, D.Griffiths, W.de Groat, and C.Fry. 2007. Origin of spontaneous activity in neonatal and adult rat bladders and its enhancement by stretch and muscarinic agonists. *Am. J. Physiol Renal Physiol* 292:F1065-F1072.

Kelly,E., C.P.Bailey, and G.Henderson. 2008. Agonist-selective mechanisms of GPCR desensitization. *Br. J. Pharmacol.* 153 Suppl 1:S379-S388.

Kennedy,C., P.N.Tasker, G.Gallacher, and T.D.Westfall. 2007. Identification of atropine- and P2X1 receptor antagonist-resistant, neurogenic contractions of the urinary bladder. *J. Neurosci.* 27:845-851.

Khatri,J.J., K.M.Joyce, F.V.Brozovich, and S.A.Fisher. 2001. Role of myosin phosphatase isoforms in cGMP-mediated smooth muscle relaxation. *J. Biol. Chem.* 276:37250-37257.

Kitazawa,T., M.Eto, T.P.Woodsome, and M.Khalequzzaman. 2003. Phosphorylation of the myosin phosphatase targeting subunit and CPI-17 during Ca2+ sensitization in rabbit smooth muscle. *J. Physiol* 546:879-889.

Kitazawa,T., S.Kobayashi, K.Horiuti, A.V.Somlyo, and A.P.Somlyo. 1989. Receptor-coupled, permeabilized smooth muscle. Role of the phosphatidylinositol cascade, G-proteins, and modulation of the contractile response to Ca2+. *J. Biol. Chem.* 264:5339-5342.

Kitazawa,T., N.Takizawa, M.Ikebe, and M.Eto. 1999. Reconstitution of protein kinase C-induced contractile Ca2+ sensitization in triton X-100-demembranated rabbit arterial smooth muscle. *J. Physiol* 520 Pt 1:139-152.

Klotz,T., B.Bruggenjurgen, M.Burkart, and A.Resch. 2007. The economic costs of overactive bladder in Germany. *Eur. Urol.* 51:1654-1662.

Lagou,M., M.J.Drake, M.Markerink-VAN Ittersum, J.DE Vente, and J.I.Gillespie. 2006. Interstitial cells and phasic activity in the isolated mouse bladder. *BJU. Int.* 98:643-650.

Ledoux,J., A.D.Bonev, and M.T.Nelson. 2008. Ca2+-activated K+ channels in murine endothelial cells: block by intracellular calcium and magnesium. *J. Gen. Physiol* 131:125-135.

Limouze,J., A.F.Straight, T.Mitchison, and J.R.Sellers. 2004. Specificity of blebbistatin, an inhibitor of myosin II. *J. Muscle Res. Cell Motil.* 25:337-341.

Lin,M.J., S.H.Liu, and S.Y.Lin-Shiau. 1998. Phorbol ester-induced contractions of mouse detrusor muscle are inhibited by nifedipine. *Naunyn Schmiedebergs Arch. Pharmacol.* 357:553-557.

Lin,V.K., J.B.Robertson, I.L.Lee, P.E.Zimmern, and J.D.McConnell. 2000. Smooth muscle myosin heavy chains are developmentally regulated in the rabbit bladder. *J. Urol.* 164:1376-1380.

Liu,W., Y.Li, S.Cunha, G.Hayward, and L.Baskin. 2000. Diffusable growth factors induce bladder smooth muscle differentiation. *In Vitro Cell Dev. Biol. Anim* 36:476-484.

Lofgren,M., E.Ekblad, I.Morano, and A.Arner. 2003. Nonmuscle Myosin motor of smooth muscle. *J. Gen. Physiol* 121:301-310.

Lorenz,K., M.J.Lohse, and U.Quitterer. 2003. Protein kinase C switches the Raf kinase inhibitor from Raf-1 to GRK-2. *Nature* 426:574-579.

Lucius,C., A.Arner, A.Steusloff, M.Troschka, F.Hofmann, K.Aktories, and G.Pfitzer. 1998. Clostridium difficile toxin B inhibits carbachol-induced force and myosin light chain phosphorylation in guinea-pig smooth muscle: role of Rho proteins. *J. Physiol* 506 (Pt 1):83-93.

Maggi,C.A., P.Santicioli, P.Geppetti, S.Frilli, M.G.Spillantini, C.Nediani, S.P.Hunt, and A.Meli. 1988. Biochemical, anatomical and functional correlates of postnatal development of the capsaicin-sensitive innervation of the rat urinary bladder. *Brain Res.* 471:183-190.

Malmqvist,U., A.Arner, R.Makuch, and R.Dabrowska. 1996. The effects of caldesmon extraction on mechanical properties of skinned smooth muscle fibre preparations. *Pflugers Arch.* 432:241-247.

Matsui,M., D.Motomura, H.Karasawa, T.Fujikawa, J.Jiang, Y.Komiya, S.Takahashi, and M.M.Taketo. 2000. Multiple functional defects in peripheral autonomic organs in mice lacking muscarinic acetylcholine receptor gene for the M3 subtype. *Proc. Natl. Acad. Sci. U. S. A* 97:9579-9584.

Matthew,J.D., A.S.Khromov, M.J.McDuffie, A.V.Somlyo, A.P.Somlyo, S.Taniguchi, and K.Takahashi. 2000. Contractile properties and proteins of smooth muscles of a calponin knockout mouse. *J. Physiol* 529 Pt 3:811-824.

Meredith,A.L., K.S.Thorneloe, M.E.Werner, M.T.Nelson, and R.W.Aldrich. 2004. Overactive bladder and incontinence in the absence of the BK large conductance Ca2+-activated K+ channel. *J. Biol. Chem.* 279:36746-36752.

Michel,M.C. and M.M.Barendrecht. 2008. Physiological and pathological regulation of the autonomic control of urinary bladder contractility. *Pharmacol. Ther.* 117:297-312.

Mills,I.W., J.E.Greenland, G.McMurray, R.McCoy, K.M.Ho, J.G.Noble, and A.F.Brading. 2000. Studies of the pathophysiology of idiopathic detrusor instability: the physiological properties of the detrusor smooth muscle and its pattern of innervation. *J. Urol.* 163:646-651.

Milsom,I., W.Stewart, and J.Thuroff. 2000. The prevalence of overactive bladder. *Am. J. Manag. Care* 6:S565-S573.

Mizuno,Y., E.Isotani, J.Huang, H.Ding, J.T.Stull, and K.E.Kamm. 2008. Myosin light chain kinase activation and calcium sensitization in smooth muscle in vivo. *Am. J. Physiol Cell Physiol*.

Morano,I., G.X.Chai, L.G.Baltas, V.Lamounier-Zepter, G.Lutsch, M.Kott, H.Haase, and M.Bader. 2000. Smooth-muscle contraction without smooth-muscle myosin. *Nat. Cell Biol.* 2:371-375.

Morrison,J., W.D.Steers, and A.Brading. 2002. Neurophysiology and neuropharmacology. *In* Incontinence. P.Abrams, L.Cardozo, S.Khoury, and A.Wein, editors. Health Publication.

Murthy,K.S., H.Zhou, J.R.Grider, D.L.Brautigan, M.Eto, and G.M.Makhlouf. 2003. Differential signalling by muscarinic receptors in smooth muscle: m2-mediated inactivation of myosin light chain kinase via Gi3, Cdc42/Rac1 and p21-activated kinase 1 pathway, and m3-mediated MLC20 (20 kDa regulatory light chain of myosin II) phosphorylation via Rho-associated kinase/myosin phosphatase targeting subunit 1 and protein kinase C/CPI-17 pathway. *Biochem. J.* 374:145-155.

Mustafa,S.M. and O.Thulesius. 1999. Cooling-induced bladder contraction: studies on isolated detrusor muscle preparations in the rat. *Urology* 53:653-657.

Ng,Y.K., W.C.de Groat, and H.Y.Wu. 2007. Smooth muscle and neural mechanisms contributing to the down-regulation of neonatal rat spontaneous bladder contractions during postnatal development. *Am. J. Physiol Regul. Integr. Comp Physiol*.

Nilsson,H. and C.Aalkjaer. 2003. Vasomotion: mechanisms and physiological importance. *Mol. Interv.* 3:79-89, 51.

Ogut,O. and F.V.Brozovich. 2000. Determinants of the contractile properties in the embryonic chicken gizzard and aorta. *Am. J. Physiol Cell Physiol* 279:C1722-C1732.

Pang,H., Z.Guo, W.Su, Z.Xie, M.Eto, and M.C.Gong. 2005. RhoA-Rho kinase pathway mediates thrombin- and U-46619-induced phosphorylation of a myosin phosphatase inhibitor, CPI-17, in vascular smooth muscle cells. *Am. J. Physiol Cell Physiol* 289:C352-C360.

Park,J.Y., H.W.Kang, H.J.Moon, S.U.Huh, S.W.Jeong, N.M.Soldatov, and J.H.Lee. 2006. Activation of protein kinase C augments T-type Ca2+ channel activity without changing channel surface density. *J. Physiol* 577:513-523.

Peng,H., V.Matchkov, A.Ivarsen, C.Aalkjaer, and H.Nilsson. 2001. Hypothesis for the initiation of vasomotion. *Circ. Res.* 88:810-815.

Persson,K., Y.Igawa, A.Mattiasson, and K.E.Andersson. 1992. Effects of inhibition of the L-arginine/nitric oxide pathway in the rat lower urinary tract in vivo and in vitro. *Br. J. Pharmacol.* 107:178-184.

Persson,K., J.J.Sando, J.B.Tuttle, and W.D.Steers. 1995. Protein kinase C in cyclic stretch-induced nerve growth factor production by urinary tract smooth muscle cells. *Am. J. Physiol* 269:C1018-C1024.

Pfitzer,G., C.Zeugner, M.Troschka, and J.M.Chalovich. 1993. Caldesmon and a 20-kDa actin-binding fragment of caldesmon inhibit tension development in skinned gizzard muscle fiber bundles. *Proc. Natl. Acad. Sci. U. S. A* 90:5904-5908.

Rasmussen,H., Y.Takuwa, and S.Park. 1987. Protein kinase C in the regulation of smooth muscle contraction. *FASEB J.* 1:177-185.

Rivera,L. and A.F.Brading. 2006. The role of Ca2+ influx and intracellular Ca2+ release in the muscarinic-mediated contraction of mammalian urinary bladder smooth muscle. *BJU. Int.* 98:868-875.

Roberts,J.A., C.Vial, H.R.Digby, K.C.Agboh, H.Wen, A.tterbury-Thomas, and R.J.Evans. 2006. Molecular properties of P2X receptors. *Pflugers Arch.* 452:486-500.

Ruggieri,M.R., Sr. and A.S.Braverman. 2006. Regulation of bladder muscarinic receptor subtypes by experimental pathologies. *Auton. Autacoid. Pharmacol.* 26:311-325.

Saitoh,M., T.Ishikawa, S.Matsushima, M.Naka, and H.Hidaka. 1987. Selective inhibition of catalytic activity of smooth muscle myosin light chain kinase. *J. Biol. Chem.* 262:7796-7801.

Sauzeau,V., J.H.Le, C.Cario-Toumaniantz, A.Smolenski, S.M.Lohmann, J.Bertoglio, P.Chardin, P.Pacaud, and G.Loirand. 2000. Cyclic GMP-dependent protein kinase signaling pathway inhibits RhoA-induced Ca2+ sensitization of contraction in vascular smooth muscle. *J. Biol. Chem.* 275:21722-21729.

SBU. 2000. Behandling av urininkontinens-sammanfattning och slutsatser av SBU:s styrelse och råd.

Schneider,T., C.Fetscher, S.Krege, and M.C.Michel. 2004a. Signal transduction underlying carbachol-induced contraction of human urinary bladder. *J. Pharmacol. Exp. Ther.* 309:1148-1153.

Schneider,T., P.Hein, and M.C.Michel. 2004b. Signal transduction underlying carbachol-induced contraction of rat urinary bladder. I. Phospholipases and Ca2+ sources. *J. Pharmacol. Exp. Ther.* 308:47-53.

Schultens,A., T.Becker, D.Balmer, D.Seidlova-Wuttke, and W.Wuttke. 2004. In vivo properties of the urinary bladder wall and their modulation by estradiol and raloxifene in a rat model. *Exp. Clin. Endocrinol. Diabetes* 112:514-519.

Sjogren,C., K.E.Andersson, S.Husted, A.Mattiasson, and B.Moller-Madsen. 1982. Atropine resistance of transmurally stimulated isolated human bladder muscle. *J. Urol.* 128:1368-1371.

Sjuve,R., T.Ingvarson, A.Arner, and B.Uvelius. 1995. Effects of purinoceptor agonists on smooth muscle from hypertrophied rat urinary bladder. *Eur. J. Pharmacol.* 276:137-144.

Smet,P.J., K.A.Edyvane, J.Jonavicius, and V.R.Marshall. 1996. Neuropeptides and neurotransmitter-synthesizing enzymes in intrinsic neurons of the human urinary bladder. *J. Neurocytol.* 25:112-124.

Somlyo,A.P. and A.V.Somlyo. 2003. Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev.* 83:1325-1358.

Straight,A.F., A.Cheung, J.Limouze, I.Chen, N.J.Westwood, J.R.Sellers, and T.J.Mitchison. 2003. Dissecting temporal and spatial control of cytokinesis with a myosin II Inhibitor. *Science* 299:1743-1747.

Szell,E.A., G.T.Somogyi, W.C.de Groat, and G.P.Szigeti. 2003. Developmental changes in spontaneous smooth muscle activity in the neonatal rat urinary bladder. *Am. J. Physiol Regul. Integr. Comp Physiol* 285:R809-R816.

Takahashi,R., J.Nishimura, K.Hirano, N.Seki, S.Naito, and H.Kanaide. 2004. Ca2+ sensitization in contraction of human bladder smooth muscle. *J. Urol.* 172:748-752.

Tansey,M.G., K.Luby-Phelps, K.E.Kamm, and J.T.Stull. 1994. Ca(2+)-dependent phosphorylation of myosin light chain kinase decreases the Ca2+ sensitivity of light chain phosphorylation within smooth muscle cells. *J. Biol. Chem.* 269:9912-9920.

Thom,D.H., M.N.Haan, and S.K.Van Den Eeden. 1997. Medically recognized urinary incontinence and risks of hospitalization, nursing home admission and mortality. *Age Ageing* 26:367-374.

Thorneloe,K.S., A.M.Knorn, P.E.Doetsch, E.S.Lashinger, A.X.Liu, C.T.Bond, J.P.Adelman, and M.T.Nelson. 2008. Small-conductance, Ca(2+) -activated K+ channel 2 is the key functional component of SK channels in mouse urinary bladder. *Am. J. Physiol Regul. Integr. Comp Physiol* 294:R1737-R1743.

Toullec,D., P.Pianetti, H.Coste, P.Bellevergue, T.Grand-Perret, M.Ajakane, V.Baudet, P.Boissin, E.Boursier, F.Loriolle, and . 1991. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.* 266:15771-15781.

Uehata,M., T.Ishizaki, H.Satoh, T.Ono, T.Kawahara, T.Morishita, H.Tamakawa, K.Yamagami, J.Inui, M.Maekawa, and S.Narumiya. 1997. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* 389:990-994.

Uvelius,B., P.O.Andersson, and A.Malmgren. 1990. Effects of variations in extracellular pH on spontaneous contractile activity and response to nerve stimulation in smooth muscle from rat urinary bladder. *Scand. J. Urol. Nephrol.* 24:47-51.

Uvelius,B. and G.Gabella. 1980. Relation between cell length and force production in urinary bladder smooth muscle. *Acta Physiol Scand.* 110:357-365.

van Koppen,C.J. and B.Kaiser. 2003. Regulation of muscarinic acetylcholine receptor signaling. *Pharmacol. Ther.* 98:197-220.

van Mastrigt,R. 2002. Mechanical properties of (urinary bladder) smooth muscle. *J. Muscle Res. Cell Motil.* 23:53-57.

Vial,C. and R.J.Evans. 2000. P2X receptor expression in mouse urinary bladder and the requirement of P2X(1) receptors for functional P2X receptor responses in the mouse urinary bladder smooth muscle. *Br. J. Pharmacol.* 131:1489-1495.

Weber,L.P., J.E.Van Lierop, and M.P.Walsh. 1999. Ca2+-independent phosphorylation of myosin in rat caudal artery and chicken gizzard myofilaments. *J. Physiol* 516 (Pt 3):805-824.

Wegener,J.W., V.Schulla, T.S.Lee, A.Koller, S.Feil, R.Feil, T.Kleppisch, N.Klugbauer, S.Moosmang, A.Welling, and F.Hofmann. 2004. An essential role of Cav1.2 L-type calcium channel for urinary bladder function. *FASEB J.* 18:1159-1161.

Werner,M.E., A.M.Knorn, A.L.Meredith, R.W.Aldrich, and M.T.Nelson. 2006. Frequency encoding of cholinergic- and purinergic-mediated signaling to mouse urinary bladder smooth muscle: Modulation by BK channels. *Am. J. Physiol Regul. Integr. Comp Physiol*.

White,S.L., M.Y.Zhou, R.B.Low, and M.Periasamy. 1998. Myosin heavy chain isoform expression in rat smooth muscle development. *Am. J. Physiol* 275:C581-C589.

Wibberley,A., Z.Chen, E.Hu, J.P.Hieble, and T.D.Westfall. 2003. Expression and functional role of Rho-kinase in rat urinary bladder smooth muscle. *Br. J. Pharmacol.* 138:757-766.

Wilson,L., J.S.Brown, G.P.Shin, K.O.Luc, and L.L.Subak. 2001. Annual direct cost of urinary incontinence. *Obstet. Gynecol.* 98:398-406.

Wu,X., A.V.Somlyo, and A.P.Somlyo. 1996. Cyclic GMP-dependent stimulation reverses G-protein-coupled inhibition of smooth muscle myosin light chain phosphate. *Biochem. Biophys. Res. Commun.* 220:658-663.

Wuest,M., N.Hiller, M.Braeter, O.W.Hakenberg, M.P.Wirth, and U.Ravens. 2007. Contribution of Ca2+ influx to carbachol-induced detrusor contraction is different in human urinary bladder compared to pig and mouse. *Eur. J. Pharmacol.* 565:180-189.

Yanai,Y., H.Hashitani, Y.Kubota, S.Sasaki, K.Kohri, and H.Suzuki. 2006. The role of Ni(2+)-sensitive T-type Ca(2+) channels in the regulation of spontaneous excitation in detrusor smooth muscles of the guinea-pig bladder. *BJU. Int.* 97:182-189.

Young,H.M., D.Ciampoli, P.J.Johnson, and M.J.Stebbing. 1996. Inhibitory transmission to the longitudinal muscle of the mouse caecum is mediated largely by nitric oxide acting via soluble guanylyl cyclase. *J. Auton. Nerv. Syst.* 61:103-108.

Zderic,S.A., J.Hypolite, J.W.Duckett, H.M.Snyder, III, A.J.Wein, and R.M.Levin. 1991. Developmental aspects of bladder contractile function: sensitivity to extracellular calcium. *Pharmacology* 43:61-68.